EVALUATION OF HYPERGLYCOSYLATED hCG AS A PREDICTOR OF ADVERSE PREGNANCY OUTCOME

NAHLA KAZIM

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Declaration

I, Nahla Kazim, declare that the following dissertation is entirely my own work. This work is original and the results presented in this thesis are my own work. All the people that have contributed advice, technique or information and that helped me in preparing this dissertation are fully acknowledged. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Nahla Kazim 19/5/10

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Abstract

Hyperglycosylated human chorionic gonadotrophin (HhCG) is a glycoprotein hormone that reportedly has biological functions different from those of hCG. It is produced by invasive cytotrophoblast cells at the time of implantation and in gestational trophoblastic disease. The invasion of cytotrophoblasts and their regulated proliferation are major determinants of pregnancy outcome: a shift of these controlling mechanisms has been associated with adverse pregnancy complications. Altered levels of HhCG may mirror placental dysfunction or impaired placental differentiation.

This observational cohort study was undertaken to correlate various pregnancy outcomes with serum and urinary concentrations of HhCG and other hCG molecular forms, in order to determine whether HhCG is a helpful predictor of adverse pregnancy outcome. The first cohort included 287 women with spontaneous conceptions who attended the Obstetrics and Gynaecology Department of Mafraq Hospital in the United Arab Emirates. Paired serum and urine samples were collected on a single occasion from singleton pregnancies between 6 and 24 weeks of gestation. Patients were followed up until the pregnancy outcome was available. HhCG levels in pregnancies with uneventful outcomes and those with pregnancy complications were compared. Significantly lower HhCG levels were observed for pregnancies resulting in miscarriage as compared with late pregnancy complications.

In the second cohort of 128 patients undergoing in vitro fertilisation or intracytoplasmic sperm injection at the Assisted Conception Unit in Edinburgh, the usefulness of serum HhCG levels on Day 14 of oocyte retrieval was assessed. HhCG levels were found to be significantly lower in pregnancies ending in spontaneous miscarriages and biochemical pregnancies. The potential diagnostic and prognostic utility of HhCG was confirmed by a receiver operating characteristic curve plot.

The sugar chain heterogeneity of hCG from various sources was also investigated by SDS-PAGE and immunoblot analyses of pregnancy urine samples to assess their potential value as new diagnostic tools for predicting pregnancy outcome. Using monoclonal antibodies and a panel of lectins that can separate N- from O-linked sugar chains, variations in the hCG glycosylation patterns during different stages of pregnancy and pregnancy complication were studied. While the differences in the hCG glycosylation profiles reported here are interesting, whether they can helpfully contribute to the prediction of pregnancy outcome requires further study.

Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
AFP	Alpha-fetoprotein
APH	Antepartum haemorrhage
AUC	Area under the curves
bFGF	basic fibroblast growth factor
BMI	Body Mass Index
CAP	College of American Pathologists
CEMACH	Confidential enquiry into maternal deaths
CHM	Classical/complete hydatidiform mole
COX	Cyclooxygenase
CTB	Cytotrophoblasts
CTP	Carboxy-terminal extension, C terminal peptide
CX	Connexin protein units
Cys	Cysteine
D	Day
DHEAS	Dehydroepiandrosterone sulphate
DM	Diabetes mellitus
DS	Down syndrome
E	Estrogens
ECM	Extracellular matrix
EECF	Extraembryonic coelomic fluid
EIA	Enzyme immunoassay
EP	Ectopic pregnancy
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinases
ET	Embryo transfer
FDA	Food and Drug Association
FGR	Fetal growth restriction
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
FSH	Follicle-stimulating hormone
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GDM	Gestational diabetes mellitus
Glc	Glucose
GlcNAc	N-acetylglucosamine
GnRH	Gonadotrophin-releasing hormone
GTD	Gestational trophoblastic disease
hCG	Human chorionic gonadotrophin
hCG α	Free alpha-subunit of hCG
hCG β	Free beta-subunit of hCG
hCGn	Nicked hCG
hCGβcf	Beta-core fragment of hCG
HG	Hyperemesis gravidarum
HhCG	Hyperglycosylated hCG
HhCGβ	Hyperglycosylated free β-subunit of hCG
HLA	Human leukocyte antigen
HM	Hydatidiform mole

HPT	Home pregnancy test
HTN	Hypertension
ICMA	Immunochemiluminometric assay
ICSI	Intracytoplasmic sperm injection
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IMFA	Immunofluorometric assay
IGFBP-1	Insulin-like growth factor binding protein-1
IL	Interleukin
IRMA	Immunoradiometric assay
IRP	International Reference Preparation
ISOBM	International Society of Oncology and Biomarkers
ITA	Invasive Trophoblastic Antigen
IU	International Unit
IUD	Intrauterine fetal death
IUGR	Intra-uterine growth retardation
IVF	In-vitro fertilization
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
MA	Missed abortion
Man	Mannose
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
MOM	Multiple of medians
MTX	Methotrexate
NGF	Nerve growth factor
nhCGβ	Nicked free β-subunit of hCG
NIBSC	National Institute for Biological Standards and Control
NIH	National Institutes of Health
NND	Neonatal death
NPV	Negative predictive value
NS	Non-significant
NT	Nuchal translucency
OR	Oocyte retrieval
P	Progesterone
P450scc	Cytochrome P450 cholesterol side-chain cleavage
PAPP-A	Pregnancy-associated plasma protein-A
PBMC	Peripheral blood mononuclear cells
PDGFβ	Platelet derived growth factor β
PGF2α	Prostaglandin F2α
PHM	Partial hydatidiform mole
PIH	Pregnancy induced hypertension
PKC	Protein kinase C
PPV	Positive predictive value
PROM	Preterm rupture of membrane
PTD	Persistent trophoblastic neoplasia
PUL	Pregnancy of unknown location'
RIA	Radioimmunoassay
ROC	Receiver operating characteristic
RR	Reference Reagent
S	Significant

Ser	Serine
SGA	Small for gestational age
StAR	Steroidogenic acute regulatory protein
STB	Syncytiotrophoblast
T3	Triiodothyronine
T4	Thyroxine
TGFβ	Transforming growth factor β
Thr	Threonine
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
TSH	Thyroid-stimulating hormone
TVS	Transvaginal ultrasound
uE3	Conjugated estriol
UKNEQAS	United Kingdom National External Quality Assessment Service
WHO	World Health Organization
α SMA	α-smooth muscle actin

CHAPTER 1. HCG AND RELATED MOLECULAR FORMS 1

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1.1 Chorionic gonadotrophin and other glycoproteins

The placental human chorionic gonadotrophin hormone (hCG) belongs to the glycoprotein hormone family including pituitary-derived luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). These hormones are each composed of two non-covalently associated α and β subunits, of which the α-subunit is common to all (Figure 1). There is considerable homology among the β subunits, particularly between the first 115 amino acid residues of the β-subunits of LH (LHβ) and hCG (hCGβ) (81%) [1,2]. HCGβ is unique in having an extra 24 amino acids on its carboxy-terminal end which confers its biological activity. Intact hCG (i.e. the α-β heterodimer) is a highly glycosylated molecule, containing oligosaccharide side chains which account for >30% of its molecular weight. Only the heterodimer is able to bind and to stimulate the hormone receptors, as the free subunits are biologically inactive.

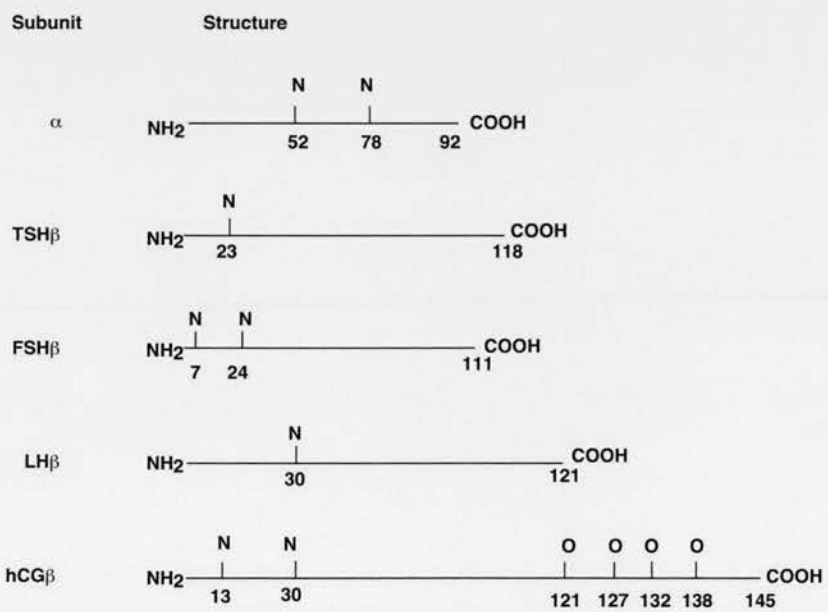

Figure 1 Schematic diagram showing the positions of N- and O-linked oligosaccharides on the α- (common) and β- (specific) subunits of glycoprotein hormones. (Figure adapted from Reference [3]).

1.2 Historical survey

The hypothalamic–pituitary–ovarian axis has been studied since the beginning of the 20th century. Early studies involved the use of hypophysectomised animal models and established that the reproductive organs were governed by the pituitary gland [4,5]. The ovarian hyperaemia and luteotrophic effects of the anterior pituitary were recognised

when anterior pituitary tissue from mice, rats, rabbits and guinea-pigs was implanted into immature female mice and rats, rapidly inducing morphological changes in their ovaries, uterus and vagina [6,7].

Other investigators of the same period also described the importance of the ovaries in the maintenance of pregnancy [for review see Reference [8]]. In 1927, Aschheim and Zondek in 1927 identified a hormone in the blood and urine of pregnant women that luteinized ovarian tissue [9]. Believing it to be produced by the anterior pituitary, they called it hypophyseal hormone ('Hypophysenvorderlappenhormone' in German). A similar substance was identified in the blood of pregnant mares [10]. It only became apparent after later experiments that the chorionic villi of the placenta rather than the pituitary was the major source of this hormonal activity [11,12], which is now known as hCG.

In the 1950s and 1960s developments in gel chromatography and isoelectric focusing enabled isolation and characterization of the four glycoprotein hormones. The subunit structure of LH was the first to be characterized by Li and Starman in 1964 [13]. Subsequent work finally established the α- and β- subunit nature and linear amino acid sequences of the peptide chains of hCG [14-16]. Since then, major efforts have been devoted to studies of the biochemistry and molecular biology of hCG, and highly sensitive techniques developed for its measurement. These studies shed light on its clinical utility in the diagnosis and monitoring of pregnancy.

1.3 Nomenclature of hCG and related molecular forms

There is considerable confusion in the hCG literature due lack of clear nomenclature. Early radioimmunoassays were termed "βhCG" assays to indicate that they were specific for hCG and did not cross-react with luteinizing hormone (LH). However this wording can be misinterpreted to mean the β-subunit of hCG. A few commercially available assays measuring both hCG and hCGβ ("total" assays) are called βhCG assays, causing further confusion. To address this problem, clear and user-friendly nomenclature has recently been developed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Table 1). This nomenclature clearly describes each major hCG variant also provides a convenient means of indicating which molecular forms an hCG assay measures (e.g. hCG or hCG+hCGβ etc). International efforts are currently under way to encourage universal adoption of this nomenclature, which has also been adopted in this thesis. In an extension to the

published nomenclature, for convenience the beta-subunits of LH, FSH and related molecules are described as LHβ, FSHβ etc throughout the present thesis.

Molecule	Symbol	Description
Intact hCG	hCG	Non-nicked hCG
Nicked hCG	hCGn	hCG missing peptide bonds in the hCGβ40-50 region
α-subunit of hCG	hCGα	Subunit dissociated from hCG
β-subunit of hCG	hCGβ	Subunit dissociated from hCG
Nicked β-subunit of hCG	hCGβn	hCGβ missing peptide bonds in the hCGβ40-50 region
β-core fragment of hCG	hCGβcf	hCGβ6-40 residues joined by disulphide bonds to hCGβ55-92

Table 1 IFCC nomenclature and hCG and other important hCG molecular forms

1.4 hCGα- and hCGβ-subunits

HCGα contains 92 amino acids and has a molecular weight of about 14,500 with carbohydrate forming approximately 30% of the weight. The subunit contains 10 cysteine residues that maintain its structural integrity by forming five disulphide bonds. The molecule also contains two N-linked oligosaccharide chains at Asn^{52} and Asn^{78} (Figure 2). Within a species, the α-subunit of hCG is able to combine with each of the other three glycoprotein β-subunits [17]

ala-pro-asp-val-gln-asp-cys-pro-glu-cys-thr-leu-gln-glu-asp-pro-phe-phe-ser-gln-pro-gly-
1 2 ↑ 3 ↑ 4

ala-pro-ile-leu-gln-cys-met-gly-cys-cys-phe-ser-arg-ala-tyr-pro-thr-pro-leu-arg-ser-lys-
↑

lys-thr-met-leu-val-gln-lys-asn-val-thr-ser-glu-ser-thr-cys-cys-val-ala-lys-ser-tyr-asn-arg-
N
52

val-thr-val-met-gly-gly-phe-lys-val-glu-asn-his-thr-ala-cys-his-cys-ser-thr-cys-tyr-tyr-his-lys-ser
N
78 92

Figure 2 Amino acid sequence of hCGα showing the carbohydrate attachment sites. Arrows represent nicking positions on the protein backbone. [18]

The beta-subunit of hCG (hCGβ) contains 145 amino acids and has a molecular weight of about 22,200, also composed of approximately 30% carbohydrate. hCGβ contains 6 disulphide bonds joining cysteines. The first 121 amino acids of the β-

subunit show 81% sequence identity with the β-subunit of LH. The sequence identity between the beta-subunit of hCG and those of FSH and TSH is 34% and 38%, respectively. HCGβ contains six oligosaccharide chains. Two of these are N-linked to Asn^{13} and Asn^{30} and the remaining four are O-linked attachment sites located on serine/proline-rich domains at Ser^{121}, Ser^{127}, Ser^{132}, and Ser^{138} (Figure 3).

Figure 3 Amino acid sequence of the β-subunit with carbohydrate (N) and (O) attachment sites. Arrow represents a nicking position on the protein backbone. (Adapted from Reference 378).

Structural elucidation of the glycans is possible after their chemical or enzymatic release from the protein backbone, followed by chromatographic separation and chemical [18-20] or mass spectrometric investigation [21, 22]. N- and O-linked oligosaccharides of hCG show structural microheterogeneity throughout normal and abnormal pregnancy as well as in trophoblastic diseases (Figure 4 and 5) [3, 18, 23-27].

N-linked oligosaccharides purified from urine of individuals with normal pregnancy are predominantly fucose-free mono- and biantennary sugar chains (Figures 4 and 5). Low levels of more highly branched, fucosylated biantennary and triantennary oligosaccharides are also present. The proportions of mono- to triantennary oligosaccharides are increased in gestational trophoblastic disease (Figure 6) as compared to normal pregnancy (Figures 4 and 5) [18, 28].

The heterogeneity of hCG glycosylation is also reflected in the degree of sialylation and core fucosylation of hCG and its free subunits. N-linked oligosaccharides vary in the presence or absence of a fucose moiety joined to the serine-linked N-acetylglucosamine, as well as in the length and composition of the branches. Evidence suggests that fucosylated structures are secreted more abundantly towards the end of

gestation [27]. Variation in the degree of sialylation of both N-linked and O-linked oligosaccharides is also seen in diabetic and Down syndrome pregnancies [18,29].

The carbohydrate composition of hCG molecular forms also differs. Oligosaccharides containing a non-reducing terminal mannose are associated with the α-subunit only, whereas oligosaccharides containing fucose are associated primarily with the β-subunit [30]. The carbohydrate composition of the α-subunit of hCG in extra-embryonic coelomic fluid (EECF) contains two N-linked glycans similar to that seen in the free hCGα but the asparagine-52 glycosylation site generally contains a single antenna of sialic acid-galactose-N-acetylglucosamine on the core oligosaccharide and a terminal mannose at the other potential branch site [31]. In addition, hCGα contains much higher amounts of N-acetylglucosamine, galactose, sialic acid, and fucose [31]. Failure to combine to form intact hCG may be due to the presence of these additional branches on the glycans of the EECF hCGα, leading to coexistence of high concentrations of uncombined free α- and β-subunits in the EECF [31]. Galactose-terminated oligosaccharides are present on the hCG α-subunit in EECF, so clearance from the circulation is slower [32,33].

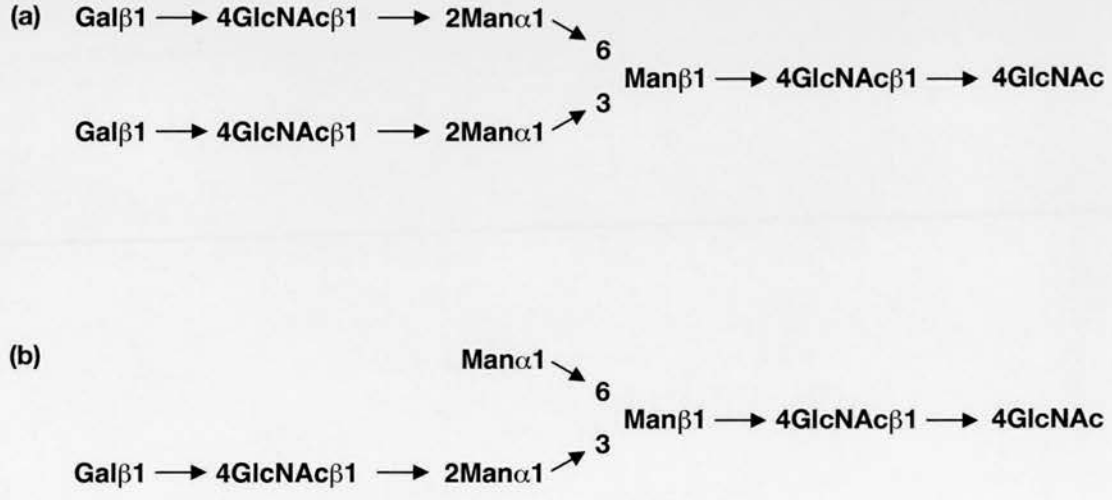

Figure 4 Fucose-free bi- (a) and mono-antennary (b) sugars of hCGα in normal pregnancy. [34]

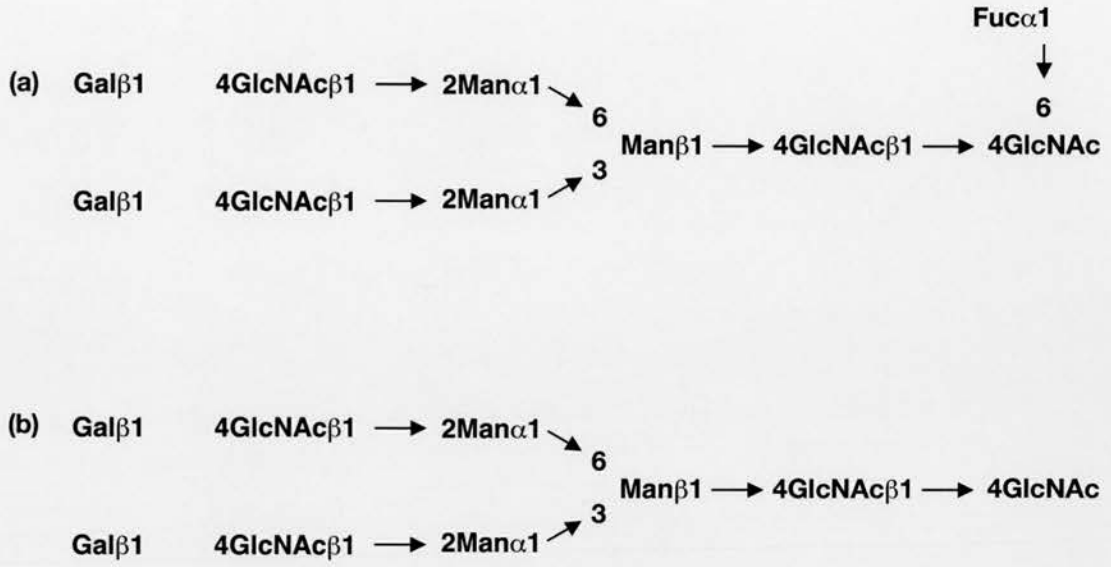

Figure 5 Fucosylated (a) and fucose free-biantennary (b) sugar chains of hCGβ from normal pregnancy. [34]

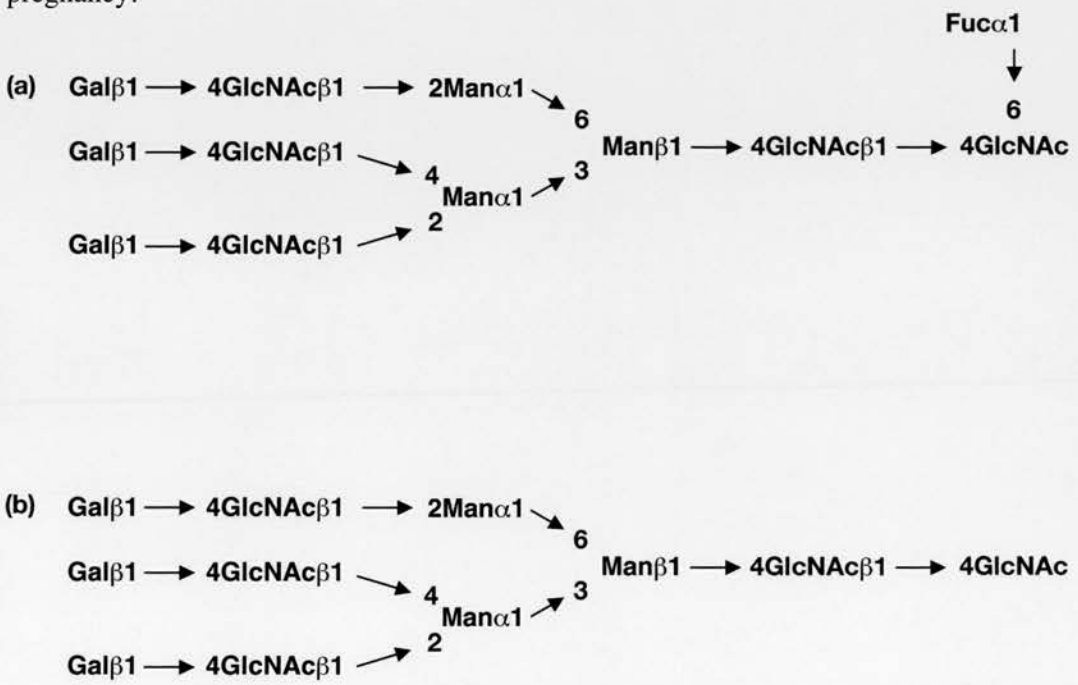

Figure 6 Fucosylated (a) and fucose-free (b) tri-and tetrasaccharide core structures found abundantly in gestational trophoblastic diseases. [34]

The major structures identified in O-linked oligosaccharides of hCGβ, isolated from the urine of healthy pregnant women and patients with trophoblastic diseases [34 18, 34], as well as the choriocarcinoma cell line [35] are galactose β1→3N-acetylgalactosamine and galactoseβ1→3N-acetylglucosamineβ1→6 N-acetylgalactosamine. Each has 0, 1, or 2 N-acetylneuraminic acids, which if present are randomly distributed among the

four serine attachment sites on the β-subunit. In normal pregnancy, the O-linked glycans contain predominantly disaccharide-core structure and significant amounts of highly branched tetrasaccharide-core structure (16%). As for N-linked oligosaccharides, the tetrasaccharide-core structure of the O-linked glycans can be more than 4.5-fold higher in individuals with choriocarcinoma, in whom levels of the disaccharide core structure may be decreased [18].

Studies of the crystal structure of hCG demonstrate that the elongated shape of the hCG molecule is roughly 75Å × 35Å × 30Å in dimension. The subunits are also elongated (approximately 60Å × 25Å × 15Å for the α-subunit and approximately 65Å × 25Å × 20Å for the β-subunit) and highly intertwined [23]. The α- and β- subunits of hCG share a remarkably similar tertiary structure. Each is held together by a set of three disulphide bridges (cysteines 10-60, 28-82, and 34-84 in the α-subunit, and cysteines 9-57, 34-88, and 38-90 in the β-subunit) from which three β-hairpin loops project.

The heterodimer is formed by the association of segments of β-sheet structures near the cystine knot of each subunit to form a short seven stranded β-barrel (Figure 7)[36, 37]. The strands likely to be involved are α52-58, α33-38, β97-102, β56-63, β84-90, β30-38, and β9-15. The heterodimer is stabilized by a segment of the β-subunit, which wraps around the α-subunit and is linked like a seatbelt by the disulphide Cys (26-100) [37]. The crystal structure of hydrofluoric acid-treated hCG reveals that the fold of both α- and β-subunits resembles the cystine-knot motif characteristic of the super-family of cystine-knot growth factors, which include nerve growth factor (NGF), transforming growth factor-β (TGFβ), and platelet derived growth factor-β (PDGFβ) [38]. These structural similarities suggest that hCG may have growth activities that have yet to be elucidated.

The disulphide bonds play a major role in stabilizing the tertiary structure of both subunits as well as the heterodimer association, which is required for full biological activity of hCG [36, 37, 39]. Much work (particularly on the β-subunit) has examined the importance of individual disulphide bonds in secretion and dimer assembly as well as their involvement in receptor binding. Site-directed mutagenesis studies and deletion of disulphide bonds by replacing cysteine with other amino acid residues, such as serine or alanine, have shown that following mutation at positions Cys 26, 110, 72 and 90 dimer assembly is reduced to <10%. [40]. Deletion of the Cys 26-100 bond

increases the rate of β-subunit secretion whereas deletion of Cys 9-90, 34-88, or 38-57 decreases it [40].

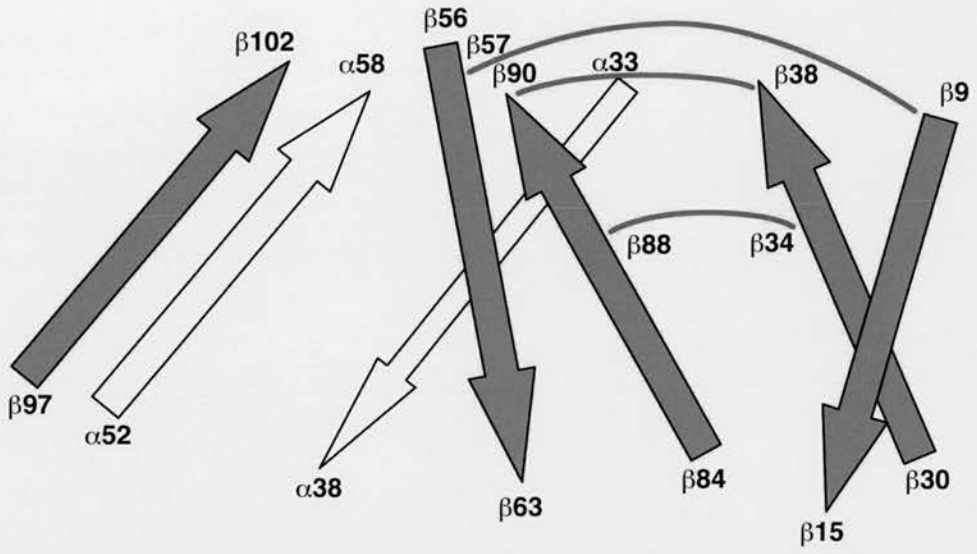

Figure 7 Schematic representation of the α- and β-subunits assembly to form hCG dimer as per the crystal structure. Red lines indicate cysteine bridges [Adapted from Reference 36]

When peptides incorporating each of the six disulphide bonds of hCGβ and their linear counterparts were screened for their ability to inhibit competitively the recombination of α- and β-subunits, it was found that disulphide peptides Cys (9-57), Cys (34-88) and Cys (38-90) inhibited αβ recombination. [36]. The remaining three disulphide peptides, [Cys (23-72), Cys (26-110) and Cys (93-100)] could not inhibit αβ dimer recombination, indicating the importance of Cys (9-57), Cys (34-88) and Cys (38-90) of the β-subunit for heterodimer formation with the α-subunit [36].

The multi-loop disulphide structure appears to provide the α-subunit with a large degree of flexibility by forming an anchoring point (with loops projecting from it in a finger-like fashion) which is able to change shape to accommodate any of the β-subunits (Figure 7) [41]. This unusual feature appears to be essential for association and stabilization of the heterodimer. Furthermore, data on the receptor binding inhibition studies using disulphide peptides of hCGβ has shown the involvement of the regions around the disulphide bonds Cys9-Cys57 and Cys23-Cys72 in receptor binding of the hormone [39].

The abundance of carbohydrate moieties on glycoprotein hormones suggests a role in heterodimer assembly and secretion. Protein glycosylation is involved in many processes including subunit folding, assembly, molecular recognition, protein

stabilization, heterodimer secretion, receptor interaction and signal transduction. N-linked glycosylation of hCG may play an essential role in signal transduction, stimulating steroidogenesis and cAMP production, but does not appear to affect the receptor binding affinity [24, 42]. Studies of the hCG folding and assembly pathway show that initial folding influences glycosylation which, in turn, influences further folding.

Elimination of two N-linked oligosaccharides from the β- subunit reduces the rate of dimer assembly, possibly by affecting the rate of folding of the subunit [24,43]. Removal of the oligosaccharide in position α52, either by site-specific mutagenesis or using chemical methods, results in increased capacity of the gonadotrophin molecules to bind to the receptor, but significantly reduces signal transduction capability. This indicates that Asn52 plays an important role in the activation of the receptor/signal transducer G protein system and hence the subsequent biological response of stimulation of steroidogenesis and cAMP production [33,43,44].

In addition, it has been shown that antibodies against hCGβ can restore the signaling activity of deglycosylated hCG, suggesting a conformational role of carbohydrates [45]. Complete removal of the glycan Asn78 in the α-subunit causes the mutant subunit to be degraded quickly and less than 20% is secreted in dimeric form. The presence of hCGβ stabilizes the α-subunit mutant and allows approximately 45% of the subunit in the form of a dimer to be secreted from the transfected cells, thus indicating the importance of glycosylation for protein stabilization [43,46]. Furthermore, elimination of Asn30 glycosylation has been shown to inhibit the secretion of uncombined hCGβ, again highlighting the importance of N-linked sugar chains of hCG in maintaining the correct conformation of each subunit [43].

Unlike the N-linked oligosaccharides, the O-linked oligosaccharides of the β subunit do not play a role either in dimer assembly or hormone secretion, but they are important for hormone bioavailability [45]. Removal of all terminal sialic acid residues and other sugar units, e.g. by chemical or enzymatic deglycosylation, makes the hCG molecule less acidic and able to form a tighter complex with the LH/CG receptor as compared to wild-type molecular forms. However, the modified hormone is unable to induce the appropriate conformational changes of the receptor required for its activation and induction of the cAMP signal-transduction pathway [47]. As a result, its ability to stimulate steroidogenesis is also hampered. Studies on the steroidogenic

activity of hCG have also demonstrated that activity declines as the length of oligosaccharide chain decreases [24, 45].

Molecular differences in hCG glycosylation or sialylation have little or no influence on the overall reactivity of hCG molecular forms or its recognition by mono- or polyclonal antibodies in assay systems. However, the absence of terminal sialic acid moieties leads to a marked loss of biological activity *in vivo*, as a result of rapid clearance of the hormone from the circulation due to the increased binding affinity of desialylated or deglycosylated hCG forms to hepatocytes [48, 49]. Heavily sialylated glycoproteins circulate for longer periods than less sialylated molecules, affecting the plasma hCG bioactivity: immunoreactivity ratio [26]. Differences in the terminal acidic or sialylated hCG molecular forms between early and late pregnancy [26, 27, 50] and between normal and abnormal pregnancy [18, 29, 51] have been shown to be influenced by various placental and/or extra-placental factors. These include the enzymatic activities of N-acetylglucosaminyl transferases and sialyl transferases, changes in progesterone concentrations and other non-steroidal factors including inhibin, activin and interleukin-6 (IL-6) [34, 52-54]. These physiological or pathological alterations in the biosynthetic pathways of hCG glycosylation may represent adaptive responses that provide the target cell with an optimal trophic signal and/ or specific hormone function.

1.5 The carboxyl-terminal extension (CTP)

HCG possesses an additional 24 amino acid carboxy-terminal extension which distinguishes it from LH. Sequence analysis demonstrates a one base pair deletion and a two-base pair insertion in the hCG gene relative to the LH coding sequence, which accounts for the extended open reading frame of hCG. There are four serine-linked oligosaccharides at residues 121, 127, 132 and 138 (Figure 8) [55].

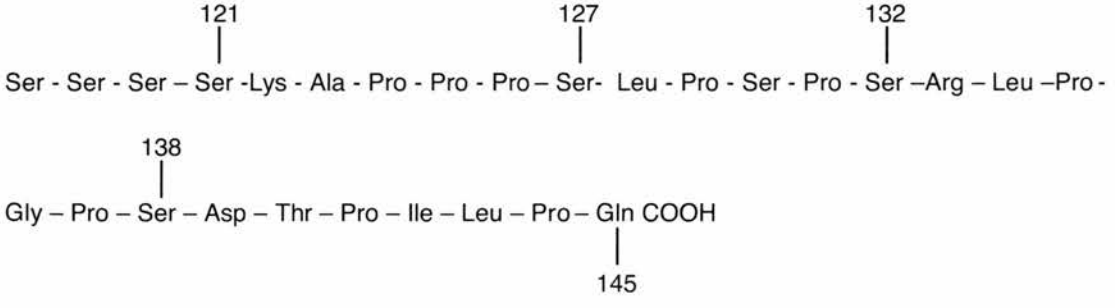

Figure 8 Amino acid sequence of the Carboxy Terminal Extension (CTP) of hCGß showing the positions of the serine linked O-oligosaccharides. [3]

The hydrophilic carboxy-terminal extension (CTP), with a repetitive sequence of proline and serine residues and containing four negatively charged O-linked oligosaccharide, has an important role in hCG bioactivity. It prolongs the circulating half-life of the hormone secondary to a decrease in renal clearance, thereby allowing it to remain in the plasma longer, with a greater likelihood of interacting with its receptor [55]. The secretion rate of a tethered form of hCG is reduced after deletion of the CTP segment [56]. Fusion of the CTP sequence of hCGβ to FSHβ significantly increased the plasma half-life and the biologic potency of the engineered chimeras [57].

In contrast to the well-established role of CTP sequence in extending the circulatory half-life of hCG, its involvement in maintaining the overall secondary/tertiary structure of hCGβ and in receptor-binding, remains controversial. Various findings support the suggestion that the CTP sequence does not contribute significantly to the overall secondary/tertiary structure of hCGβ, nor does it appear to be involved in receptor-binding [46, 55]. These include the fact that the native hCGβ or the synthetic CTP peptide compete for monoclonal antibodies (MAbs) to the CTP at the same molarity with radio-labelled denatured hCG, the ability of the hCG carboxy-terminal mutants to combine efficiently with α-subunit, the ability of CTP sequence to be fused to the FSHβ without affecting the signal transduction of the chimera, and the inability to resolve the CTP region by X-ray diffraction.

The CTP can affect the intracellular behavior of the subunit by altering the post-translational processing of the mutated β-subunit [55]. Muyan et al. showed that the CTP participates in the folding of the newly synthesized subunits, as indicated by the post-translational changes in processing of the N-linked carbohydrate-processing pathway, and deletion of the C-terminal extension sequence, resulting in decreased assembly of the truncated hCGβ subunit (hCGβ114) with the α-subunit and increased heterogeneity of secreted forms of the uncombined subunits synthesized in transfected Chinese hamster ovary (CHO) cells [55].

A single chain derivative of hCG with the C-terminus of the α-subunit translationally fused to the N-terminus of the β-subunit has an overall conformation similar to that of heterodimeric hCG and binds to the LH receptor [58]. However, the mutant single chain hCGαβ lacking five C-terminal amino acids of the β-subunit shows a significant reduction in its ability to elicit full biological response [58]. There is an intra-molecular interaction between the CTP and the proximal domains of the subunits as

demonstrated by generation of an hCGβ mutant devoid of the native O-linked acceptor sites [59]. Glycosylation occurs at Ser130 in the CTP and the carbohydrate O-glycosyl units differ from those in the wild type subunit [59]. This suggests that CTP participates in the folding of newly synthesized subunits through site-specific, post-translational modifications. Despite the close relationship between the LHβ and hCGβ subunits, their modes of secretion are different and are programmed by a carboxyl-terminal sequence, which acts as a routing signal for the apical release of hCG from the placenta to the maternal circulation [60].

1.6 hCG-related molecular forms

1.6.1 Hyperglycosylated hCG

HCG produced in choriocarcinoma or gestational trophoblastic tumors contains larger triantennary N-linked oligosaccharides and hexasaccharide O-linked oligosaccharides than does hCG produced during most of normal pregnancy. During pregnancy, hCG contains primarily mono- and biantennary N-linked oligosaccharides, tri- and tetrasaccharide-type-O-linked sugar units(i.e. more complex Core 2 than Core 1 O-linked sugars) [3, 18, 28, 47, 49, 61].

The term hyperglycosylated was originally applied to molecular forms of hCG in which there is excessive branching of the N- and O-linked oligosaccharide side chains. Due to the presence of additional sialyl-N-acetylgalactosamine antennae, HhCG hCG is approximately 14% larger than hCG (molecular weight of 41,000 vs. 36,700 Daltons) [18]. More recently, the term HhCG has been used for a distinct structural variant of hCG which was generated against one choriocarcinoma hCG preparation (hCG Batch C5). [49] This immunogen contained, in addition to larger tri- and tetra-antennary N-glycans, only Core-2 O-glycan structures and none of the Core 1 glycans predominantly found in hCG prepared from mid-pregnancy urine (Figure 9) [49, 62, 63].

While various forms of HhCG have been reported (Figure 9), characterization of B152 antibody indicates that it specifically recognizes hexasaccharide O-linked oligosaccharides attached to Ser 132 on the C-terminal peptide of choriocarcinoma hCG. Assays using this antibody therefore only measure hCG molecular forms possessing a Core 2 type of branched O-glycan on this portion of hCGβ (Figures 9 and 10) [63, 64].

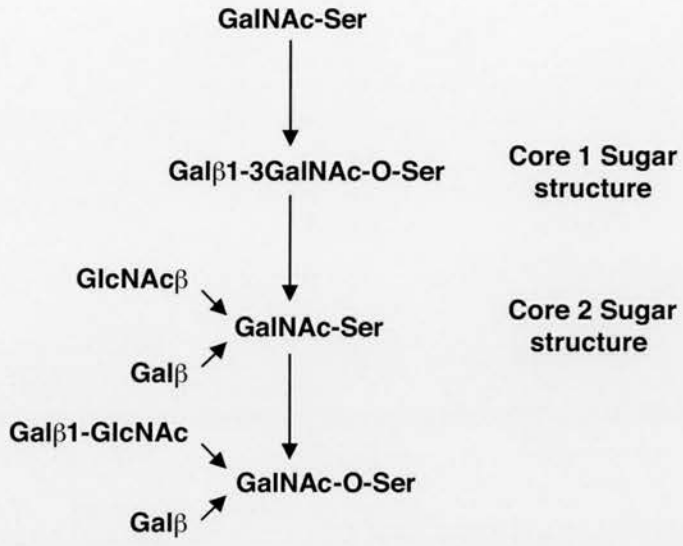

Figure 9 Schematic diagram showing the O-glycan of Core 1 sugar of normal pregnancy urine hCG and the Core 2 sugar mainly produced weeks following implantation and GTD. [63].

In an important study in 1998, HhCG was shown not only to be the principal form of hCG produced in choriocarcinoma, but also the major form produced in the weeks following normal implantation [65]. Several later publications confirm that HhCG predominates, in serum and urine, for the first six weeks of gestation, after which its levels rapidly diminish. It is replaced by other hCG molecular forms, both in spontaneous conceptions and pregnancies achieved following in vitro fertilisation (IVF) treatment [66-69].

A cellular origin of HhCG has been identified by analysis of placental cell-conditioned media. Studies have shown HhCG to be predominantly produced by cytotrophoblasts, which are the principal cells in blastocysts at the time of implantation, as well as by poorly differentiated trophoblast cells in choriocarcinoma cell-lines [70]; these studies suggest that maternal HhCG is a marker of trophoblast invasion and HhCG production is directly linked to ongoing trophoblast cell invasiveness [71-74]. Some authors therefore refer to HhCG as invasive trophoblast antigen [69, 75, 76]. This may, however, be a misnomer since HhCG is also produced by some germ cell tumours, as well as by other non-trophoblastic tumors [18, 28, 61, 77, 78].

This has encouraged studies investigating the usefulness of HhCG as a marker to detect pregnancy disorders related to defective extravillus trophoblast invasion such as early pregnancy loss, spontaneous miscarriage, pre-eclampsia and Down syndrome [67, 71, 79-81].

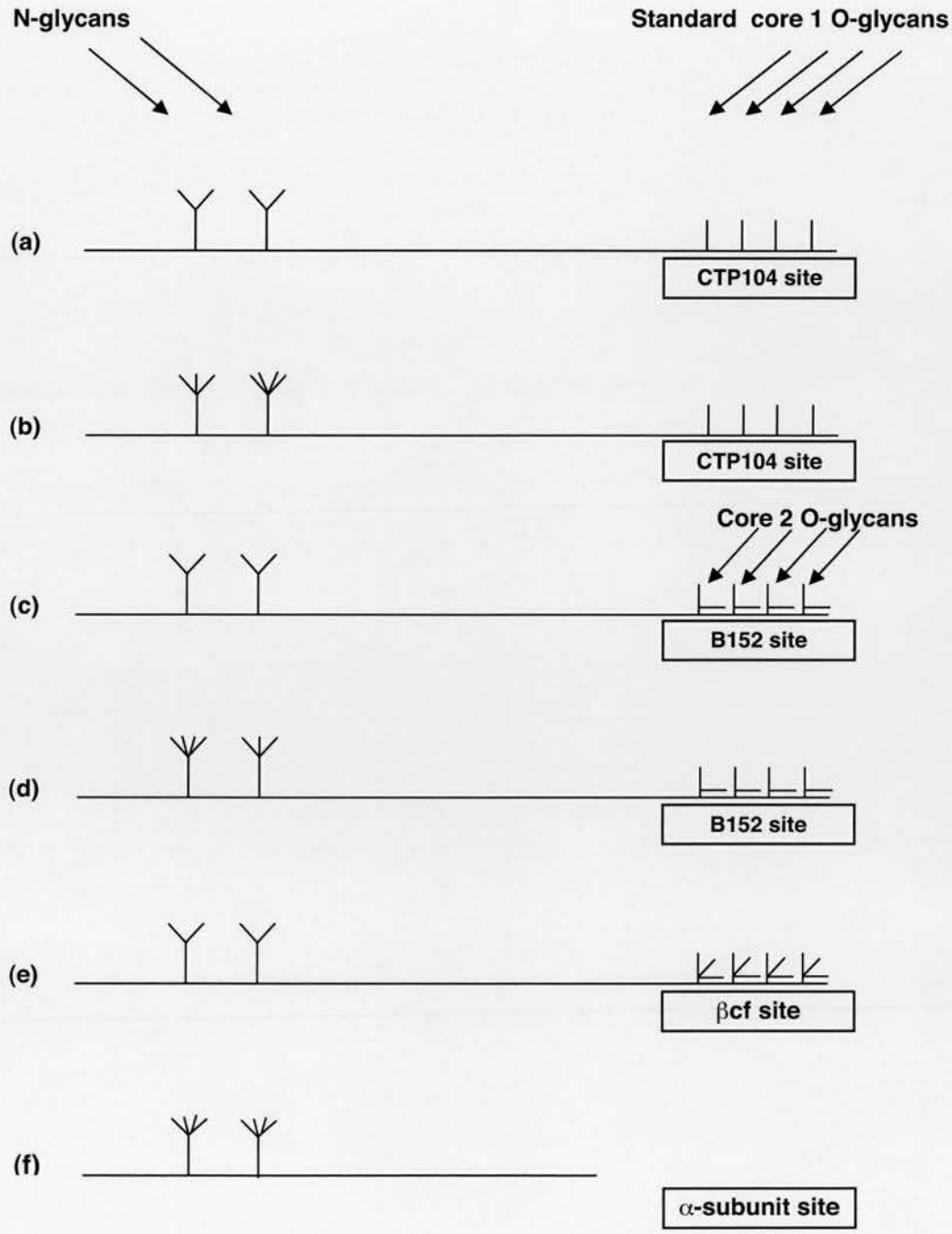

Figure 10 Schematic diagram illustrating possible hyperglycosylation sites of hCG. (a) β-subunit glycan of standard mid-trimester hCG, containing mainly Core 1 sugar chains; (b) β-subunit glycan with hyperglycosylation of N-glycan in normal pregnancy [18]; (c) hyperglycosylated Core 2 O-glycan; (d) hyperglycosylation at N- and O-linked glycans; (e) hyperglycosylation of hCGβcf [82]; (f) hyperglycosylation on the N glycan of the α-subunit [64]. [Figure adapted from Reference 63.]

1.6.2 Other hCG-related molecular forms

There are a number of other molecular forms of hCG in which the carbohydrate or protein moieties are modified. Properties of those currently considered to be most clinically relevant are summarized in Table 2.

Molecule	MW	α-subunit structure	β-subunit structure
hCG	37000	92 amino acids, no cleavages Mono- & biantennary N-linked oligosaccharides	145 amino acids, no cleavages. Biantennary N-linked ± fucose, commonly tri-& tetrasaccharide O-linked oligosaccharides
Nicked hCG	36000	92 amino acids, no cleavages Mono- & biantennary N-linked oligosaccharides	145 amino acids, cleaved at β43-44, β44-45 or β47-48. Biantennary N-linked ± fucose, commonly tri-& tetrasaccharide O-linked oligosaccharides
Hyperglycosylated hCG	41000	92 amino acids, no cleavages Mono- & biantennary + fucose N-linked oligosaccharides	145 amino acids, no cleavages. Bi- and tri-antennary N-linked ± fucose, commonly hexasaccharide O-linked oligosaccharides
Free β-subunit	22000	Absent	145 amino acids, no cleavages biantennary N-linked ± fucose, commonly tri-& tetrasaccharide O-linked oligosaccharides
Nicked free β-subunit	22000	Absent	145 amino acids, cleaved at, β43-44, β44-45 or β47-48. Biantennary N-linked ± fucose, commonly tri- and tetra-saccharide O-linked oligosaccharides
Hyperglycosylated free β-subunit	26000	Absent	145 amino acids, no cleavages Bi- and tri-antennary N-linked ± fucose, commonly hexasaccharide O-linked oligosaccharides
hCG β-core fragment	10000	Absent	Two peptides (6-40) β subunit residues linked to 55-92 degraded biantennary N-linked & no O-linked oligosaccharides
Free α-subunit	14500	92 amino acids, no cleavages. Mono- & biantennary N-linked oligosaccharides	Absent

Table 2 Major molecular forms of hCG in serum and urine [83, 84].

1.7 Synthesis and release of hCG

HCGα is encoded by a single gene on chromosome 6q21.1-23 and the specific β-subunit is encoded by a cluster of genes consisting of six hCGβ subunit genes, one hCGβ pseudogene and one LHβ gene, on chromosome 19q13.3 [85]. The synthesis of hCG starts at very early stages of pregnancy. Initiation of hCG transcription was previously observed between the six- to eight-cell stage using *in situ* hybridization [85]. In a study using the more sensitive reverse transcriptase polymerase chain reaction

(RT-PCR) technique, expression was detected as early as the two-cell stage [86]. It is generally suggested that subunit assembly and formation of the heterodimer proceed in two phases. The first involves pairing of the subunits in the lumen of the endoplasmic reticulum, with the aid of one or more chaperone molecules, and the second involves configuration of the final dimeric subunit [87-89]

The major steps in hCG biosynthesis are summarized in Figure 11 [33, 90] and involve the following:

1. Cleavage of signal peptide, by a microsomal endopeptidase, in membranes of the endoplasmic reticulum.
2. Chaperone-assisted protein folding, formation of disulphide bonds, and refolding of mis-folded protein.
3. Stepwise glycosylation which begins in the rough endoplasmic reticulum with co-translational transfer of a dolichol-linked oligosaccharide precursor to asparagine residues 52 and 75 of hCGα and 13 and 30 of hCGβ.
4. Further modification of the above precursor by glucosidases (exoglucosidases I and II) and by a mannosidase, generating high mannose intermediates composed of two N-acetyl glucosamine residues linked to three mannoses.
5. Additional trimming of glucose and mannose residues in the endoplasmic reticulum and cis-Golgi membranes.
6. Extensive processing of the oligosaccharides attached to the protein core of the hormone in the medial and trans-Golgi membranes, where N-acetylglucosamine, fucose, galactose, and sialic acid residues are added sequentially to form the mature oligosaccharides.
7. Subunit assembly accompanied by conformational changes in both subunits, which stabilize hCG.

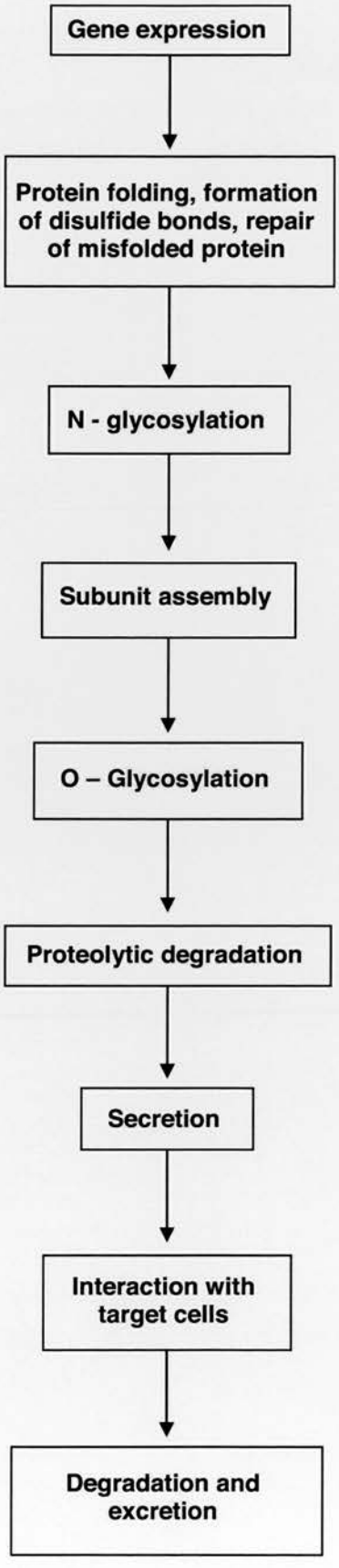

Figure 11 Schematic representation of the steps involved in hCG biosynthesis and degradation.

It is proposed that αβ dimer formation involves two pathways [91]. In the 'wraparound' pathway the α- and β-subunits initially dock before the 'seatbelt' is latched, forming a loose complex. This undergoes subsequent association-dependent folding (i.e. the seatbelt is wrapped around loop α2 forming the disulphide latch) to form the stable biologically active dimer (Figure 12a). In the 'threading' pathway, the seatbelt latch disulphide forms before the subunits dock. Assembly of the heterodimer is completed when loop α2 and its attached oligosaccharide traverses the hole in the β-subunit beneath the seatbelt (Figure 12b). The association of the subunits follows second-order kinetics and their final folding follows first-order kinetics [91]. Association is not only a necessary precondition of the formation of the biologically active hormone, but also protects the β-subunit from proteolytic digestion. Interchain hydrogen bonding is important at the subunit interface.

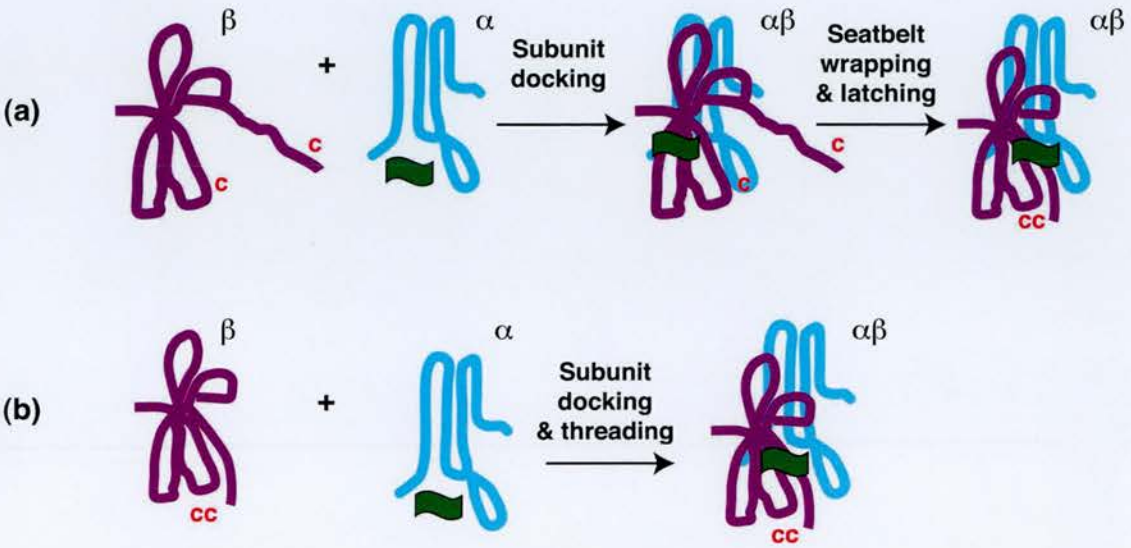

Figure 12 Pathways of α and β-subunit assembly. In the wraparound pathway (a) both subunits are initially docked before the seatbelt (i.e. formed by Cys 26-110 shown as cc in red) is latched. In the threading pathway (b) the disulphide seatbelt latches before the docking and subunit assembly. The green rectangle represents loop 2 oligosaccharide on the α-subunit involved in subunit assembly and their folding. [Figure adapted from Reference [91]]

The villous syncytiotrophoblast of the placenta is the main site of hCG production [85, 92] whereas the invasive cytrophoblasts are the primary source of HhCG [70]. The cytotrophoblast (undifferentiated stem cells) is dominant in early gestation and the syncytiotrophoblast (differentiated trophoblast transformed from cytotrophoblast) is dominant later in pregnancy [85, 92]. hCG synthesis depends on the rate of differentiation of cytotrophoblasts into syncytiotrophoblasts. [92]

In vitro studies demonstrate that cytotrophoblast cells, isolated from term placenta and cultured in maternal serum, progress to an advanced stage of trophoblast differentiation (i.e. multinucleated syncytial structures) and actively secrete hCG [93]. Cytotrophoblasts of the placenta secrete hCG on 7-9 days after conception while after implantation the placental syncytiotrophoblast starts to produce large amounts of hCG, of which more than 90% is released into the maternal circulation.

Serum hCG concentrations increase at an exponential rate during normal pregnancy, with a doubling time of up to two days for the first 5-6 weeks, reaching peak concentrations of approximately 100,000 U/L between the 7^{th} and 10^{th} weeks of gestation. After 11 weeks hCG concentrations decrease 10-fold before reaching a plateau at which they remain relatively constant until near to term, when they increase slightly before falling rapidly to within the postpartum reference range [94,95].

Histological and immunohistochemical studies demonstrate cells with many cytoplasmic protrusions containing numerous secretory granules which produce hCG [96]. These small granules are seen in the Golgi complex and migrate toward the cell surface, increasing in size by fusing with each other. They gather in cytoplasmic protrusions and following liquefaction are liberated by exocytosis into the maternal bloodstream. These hCG secretory granules arise synchronously on the surface of almost all chorionic villi. They form only at 8-9 weeks of gestation, coinciding with the maximal hCG concentration of maternal serum during pregnancy.

The rate of secretion of hCG is largely determined by synthesis of hCGβ which peaks during the first trimester, whereas concentrations of hCGα show a progressive increase throughout pregnancy. Hoshina *et al* localized hCGα mRNA in the syncytial layer and also in differentiating cytotrophoblasts and suggested that the transcription of these genes is initiated before the completion of syncytial formation, becoming attenuated as gestation progresses [97]. HCG is primarily localized to cytotrophoblasts in 4- 5 week placentas, whereas in 6-12 week placentas these substances are exclusively localized in the syncytiotrophoblast [85]. Many other studies confirm that the changing pattern of hCG secretion depends on changes in the distribution of cytotrophoblastic- and syncytiotrophoblastic-cells. This differs from the pattern observed for other placental products (e.g. progesterone and human placental lactogen), all of which show a progressive rise throughout pregnancy and, in some, a plateau towards term [98-100].

Several studies have been conducted to explain why a peak of hCG occurs early in human pregnancy. Most of the published data focus on the role of gonadotrophin-releasing hormone (GnRH), which is synthesized mainly by the cytotrophoblast and stimulates release of hCG *in vitro* [101, 102]. Pulsatile secretion of hCG has been measured in first trimester trophoblast explants to which GnRH-I or GnRH-II had been added [103]. GnRH-I induced both hCG synthesis and secretion, thus behaving as a placental GnRH, whereas GnRH-II induced hCG secretion only. The difference in actions of the two GnRHs might be due to their differential expression during phases of pregnancy, giving rise to the different pattern of hCG secretion. This may be possible since GnRH receptor mRNAs have been detected in the human placenta and are localized to the cytotrophoblast and syncytiotrophoblast cell layers [104]. Dynamic changes in the number of these mRNA levels in the placental trophoblast cells mirror the placenta's morphological transformation at various gestation ages, regulating hCG secretion during pregnancy. There is also evidence for a peak of GnRH receptors in the placenta at the end of the first trimester [105].

HCG regulates its own synthesis in the human placenta by several mechanisms. The trophoblast has surface receptors for hCG, and binding of hCG to these receptors down-regulates its own secretion (autocrine action) (Figure 13) [106, 107]. HCG can also increase cAMP levels in human placenta, and exogenous cAMP stimulates placental hCG biosynthesis and promotes differentiation of cytotrophoblasts, thereby regulating their synthesis of hCG (paracrine action). These findings suggest that cAMP may be a second messenger through which hCG can regulate its own synthesis (Figure 13) [107, 108].

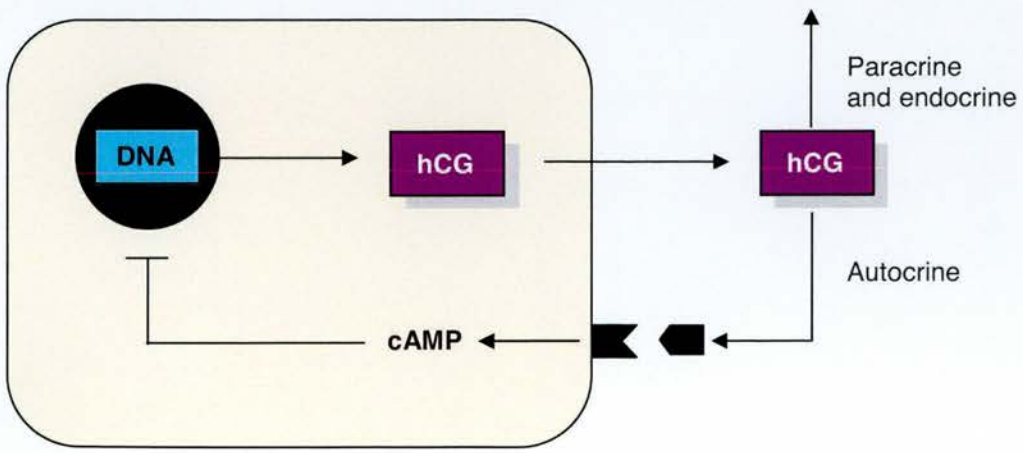

Figure 13 Factors involved in the regulation of hCG secretion. [Figure adapted from Reference 107.]

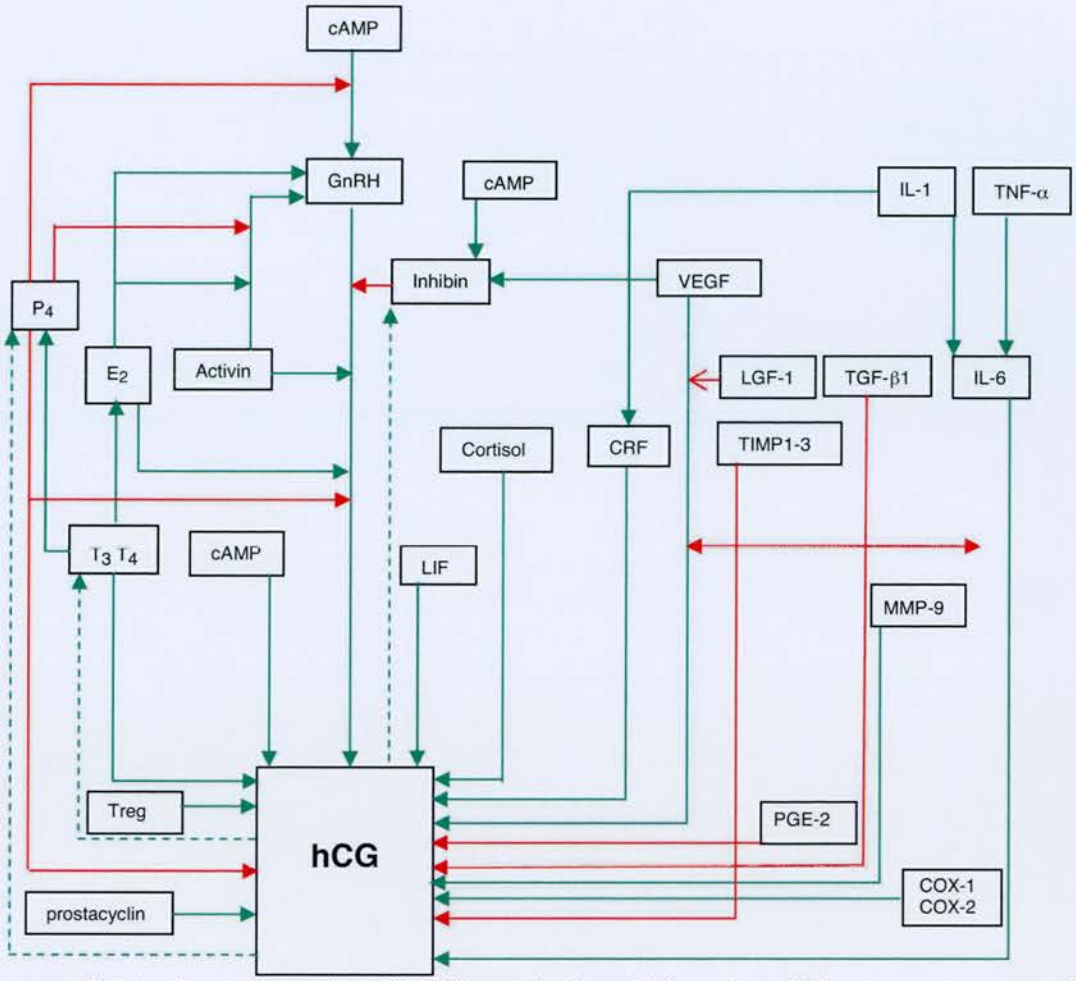

Figure 14 Stimulatory (green) and inhibitory (red) activities of possible secretagogues on the secretion of hCG.[Adapted and updated from Reference [109]]

Although it is unlikely that all pathways are functional at the same time, Figure 14 illustrates the complexity of the interactions that may occur *in vivo*. The fact that so many possible regulators of hCG secretion act in an autocrine and paracrine manner adds further complexity to this system. The imbalance between the concentrations of mature hCG vs. free subunits (e.g. in malignancy and ectopic pregnancy) and the occurrence of aberrant molecular forms (e.g. carbohydrate molecular forms) and partially degraded molecules raise a question as to how the individual steps of hCG biosynthesis are coordinated and whether they are regulated separately. Further investigation of the mechanism of hCG synthesis and secretion in pregnancy-related disorders should help to improve understanding of these complex phenomena.

1.8 Metabolism of hCG-related molecular forms

HCG in the circulation has a bi-exponential plasma half-life, with a fast disappearance half-life of approximately 5 hours and a slow half-life of approximately 35 hours [110].

The α- and β-subunits also demonstrate biphasic disappearance curves, but they are cleared from circulation much more rapidly. hCGα has a rapid half-life of 13 minutes and a slow half-life of 76 minutes whereas the corresponding half-lives for hCGβ are 41 and 236 minutes, respectively [111]. Only 25% or less of the hormone is excreted in the urine, with the remainder catabolized by the liver, kidneys and other tissues [112].

HCGβcf is the major urinary degradation production of hCG and its β-subunit. Although much of the carbohydrate present in hCGβ is missing from hCGβcf, its concanavalin-A-binding activity is retained due to the presence of a fucose core. During pregnancy hCGβcf is the major immunoreactive component of hCG in urine, with production of up to 5 mg/day and urinary concentrations 2 to 10 times higher than that of hCG on a molar basis. HCGβcf emerges as the dominant form only during the fifth week after conception [113]. Using a highly sensitive assay, very low concentrations of hCGβcf [from 2.9-34 pmol/L (i.e. < 0.1% of the concentration found in urine)] have been demonstrated in the serum of pregnant women [114]. Its high urinary concentration suggests that hCGβcf arises from hCG metabolism in the kidney [114, 115].

A study involving intravenous injection of recombinant hCG into healthy, non pregnant women showed that hCGβcf measured in urine accounted for only 12.2% of the total of hCG immunoreactivity [116]. There is some evidence of formation of hCGβcf by partial proteolysis of hCG in the tissue where hCG or hCGβ is synthesized [117, 118]. hCGβcf has also been found in the urine of men, non-pregnant women, and patients with trophoblastic disease and non-trophoblastic malignancies [118-120].

HCGn (Table 1) is also found in urine and blood of pregnant women and women with trophoblastic disease (Figure 15) [121].

Figure 15 Amino acid sequence of hCGβ showing the inter-cysteine loop β38-57 which contains the major sites of missing peptide linkages or nicks. [121]

HCGn is less stable in serum and urine than hCG and rapidly dissociates to hCGβn and hCGα [122]. It has been suggested that free hCG subunits may be generated by direct dissociation of hCG and that 'nicked' forms are dissociated into free forms and degraded into hCGβ minus CTP and hCGβcf [123, 124]. The hCG degradation pathways are thought to be more active in abnormal pregnancies, during parturition and in gestational trophoblastic disease [115, 119, 121, 125]. Studies have shown very little biological activity of nicked forms of hCG with up to 80% loss of hormone function as well as variations in *in vitro* receptor-binding activity [122, 126].

Hyperglycosylated free α- and β-subunits of hCG are also reportedly present in urine samples of pregnant women and patients with trophoblastic disease [18, 83, 115]. HhCG is believed to break down more rapidly than normally glycosylated hCG. This might be due to steric effects preventing combination of the larger hyperglycosylated free α- and β-subunits [115]. The presence of terminal sialic acid residues on each of the large carbohydrate side-chains, is also known to affect the *in vivo* half-life of hCG [127].

1.9 hCG profiles during pregnancy

Progressive changes in hCG molecular forms throughout pregnancy have been documented in many studies. On the basis of gel chromatography results, Fien *et al.* reported that first trimester hCG was more highly glycosylated than hCG found in the third trimester [128]. Wide and Hobson, measured the median charge and the ratio of bioactive to immunoreactive (B/I) ratio of hCG in serum and concluded that hCG from early pregnancy and choriocarcinoma has identical median charge with hCG from later pregnancy as well as an increased metabolic clearance rate [25]. However the same authors later demonstrated that a change in the hCG isoform distribution pattern occurs at around the 13th week of gestation [50].

Kovalevskaya et al. demonstrated progressive changes of the choriocarcinoma-like hCG isoform from 1 to 4 weeks post embryo transfer which continue through the remainder of pregnancy [66]. Several consecutive reports on HhCG in spontaneous conceptions further confirm that highly branched hCG molecular forms, produced by the invasive cytotrophoblasts, predominate during the first six weeks of pregnancy and account for up to 100% of early pregnancy hCG-related molecules). These molecular forms rapidly disappear as pregnancy progresses, and are replaced by less highly branched hCG glycoforms of mature pregnancy, so they form only a minor

component of hCG-related molecules (<2%) from 8 weeks of gestation until term [67, 68, 129, 130].

In contrast, Diaz-Cueto *et al* showed that hCG molecular forms become less acidic as gestation progresses, with most acidic forms found in samples taken at 10-11 weeks of gestation [26]. Skarulis *et al* reported that the glycosylation patterns of both hCG and hCGα change, as pregnancy progresses, due to increased branching and fucosylation [27]. These results imply that the activities of placental fucosyl transferase as well as N-acetylglucosaminyl transferases IV and V may be increased later in gestation. The latter two studies contradict the three reports described above [25, 66, 128], as they suggest that in normal pregnancy hCG increases in carbohydrate complexity and becomes less acidic as gestation progresses, i.e. begins to resemble a choriocarcinoma-like hCG isoform. Indeed, it has been suggested that enzymatic changes in early gestational trophoblast cells lead to the biosynthesis of highly branched N-linked oligosaccharides, and may explain the presence of higher proportions of HhCG during early gestation [131].

Production of the hyper-branched Core 2 glycan structure, to which MAb B152 is mainly bound, is attributed to the activity of a glycotransferase enzyme which is also active in trophoblastic tumors and cells that participate in the immune response. Such modifications of hCG during the early stage of placental development may indicate a role in trophoblast invasiveness and thereby implantation [71, 72, 132]. In contrast, the decrease in HhCG levels observed as pregnancy progresses parallels the syncytialization of the trophoblast with advancing gestation. During this process, it has been suggested that differentiated syncytiotrophoblasts secrete well-processed glycosylated molecular forms of hCG and fewer choriocarcinoma-like hCG molecular forms. Hence, alteration of protein synthesis pathway may occur concurrently with the appearance and the disappearance of a particular trophoblastic cell with advancing gestational age [20, 27, 67, 128].

Nicked hCG molecules account for approximately 9% of serum hCG molecules at the second month of gestation, the mean proportion rising to 21% of hCG molecules in the 9th month of normal pregnancy. [126] HCGα is present at low concentrations in maternal blood by the sixth week of pregnancy, and its concentration increases gradually to a maximum of about 500 μg/L [133]. During the 3rd trimester, free α-subunits constitute 30-50% of the total hCG in maternal blood. HCGβ concentrations,

like those of hCG, peak at around the 10th week of gestation, but are very low, representing only 0.9% of the total hCG concentration in the 2nd month gestation, and declining to 0.5% in the 9th month. HCGβ concentrations are initially lower than hCG concentrations but begin to increase sharply at 5 weeks of gestation, and by 6-7 weeks of gestation they are similar to urinary hCG concentrations (mole for mole), and increase from then on [83, 115, 134].

1.10 The physiological role of hCG in human pregnancy

Although an embryo is considered as a semi-allograft in the maternal environment, as half of its genetic material comes from the father, it is well tolerated due to complex biochemical and molecular mechanisms that establish a dialogue at the fetomaternal interface, thus allowing its implantation and development in the uterus. HCG plays several key roles in the apparent immunological paradox that enables survival of the semi-allogenic fetus in the maternal environment.

1.10.1 Involvement of hCG in rescue of the corpus luteum

HCG plays an important role in early pregnancy by preventing luteolysis and extending the functional life of the corpus luteum for a number of weeks, providing adequate progesterone secretion by the corpus luteum to maintain pregnancy. In primates, ovariectomy or removal of the corpus luteum prior to the luteal-placental shift causes abortion unless progesterone is replaced [135]. Exogenous administration of hCG to non-pregnant women and primates to mimic the *in vivo* pattern of hCG secretion extends the functional life span of the corpus luteum and prolongs progesterone secretion. This critical "rescuing" role of hCG is evident as anti-hCG antiserum administered during early pregnancy results in abortion in primates [136] and in a fall in progesterone level with subsequent menses in women [137].

The timing of hCG secretion and its continuous increase as the trophoblast develops is mandatory for luteotropic support of the corpus luteum. In a study assessing the response of the corpus luteum to different exogenous hCG concentrations during the mid-luteal phase of the menstrual cycle, failure of the corpus luteum was observed when hCG was injected slowly in a suboptimal manner on Day 8. [138] This mimics the hormonal environment of a failing embryo, which is characterised by steadily falling levels of progesterone. Regimes mimicking the situation in normal spontaneous pregnancies, by giving exogenous hCG starting on Day 7, rescue the corpus luteum

and induce a sustained increase in plasma progesterone from Day 8 to Day 11. [138] Plasma concentrations of progesterone fall within 24h of the last hCG injection despite the presence of residual circulating hCG [138]. The luteotropic action of hCG is mediated by 1) prevention of hCG receptor down-regulation, 2) prolonged expression of steroidogenic acute regulatory protein, 3) cytochrome P450 cholesterol side-chain cleavage, and 4) prolonged expression of 3β-hydroxysteroid dehydrogenase, all of which are required for steroidogenesis. [139]. In addition, hCG has anti-apoptotic activity in the late luteal phase, preventing luteolysis. This is achieved by 1) maintaining luteal blood flow through increased expression of angiogenic factors [140], 2) limiting tissue remodeling by controlling the activity of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) and the influx of macrophages [141], and 3) preventing stromal fibroblasts from undergoing apoptosis by decreasing the pro-apoptotic protein, Bax, and increasing the Bcl-2/Bax ratio [142, 143]. All these functions are maintained until the luteal-placental shift occurs at approximately 7 to 9 weeks of gestation, after which time the functional capacity of the corpus luteum decreases, and placental production of progesterone begins.

1.10.2 Effects of hCG on implantation

HCG also plays an important role in implantation by modulating the uterine micro-environment to allow trophoblastic invasion and maintenance of pregnancy. The process of implantation begins about 6-7 days following fertilization and consists basically of three stages (Figure 16) [144]. The first stage is the initial adhesion of the blastocyst to the uterine wall. At this stage, microprotrusions from the apical uterine epithelium surface (pinopodes) extend onto microvilli on the apical syncytiotrophoblast surface of the blastocyst. The next step is adhesion with increased physical contact between the blastocyst and the uterine epithelium and the concomitant orientation of the embryonic pole towards the luminar epithelium. The final invasive stage concludes with penetration of the syncytiotrophoblasts through the uterine epithelium to invade the entire endometrium, the inner third of the myometrium and the uterine vasculature, thus establishing uteroplacental circulation.

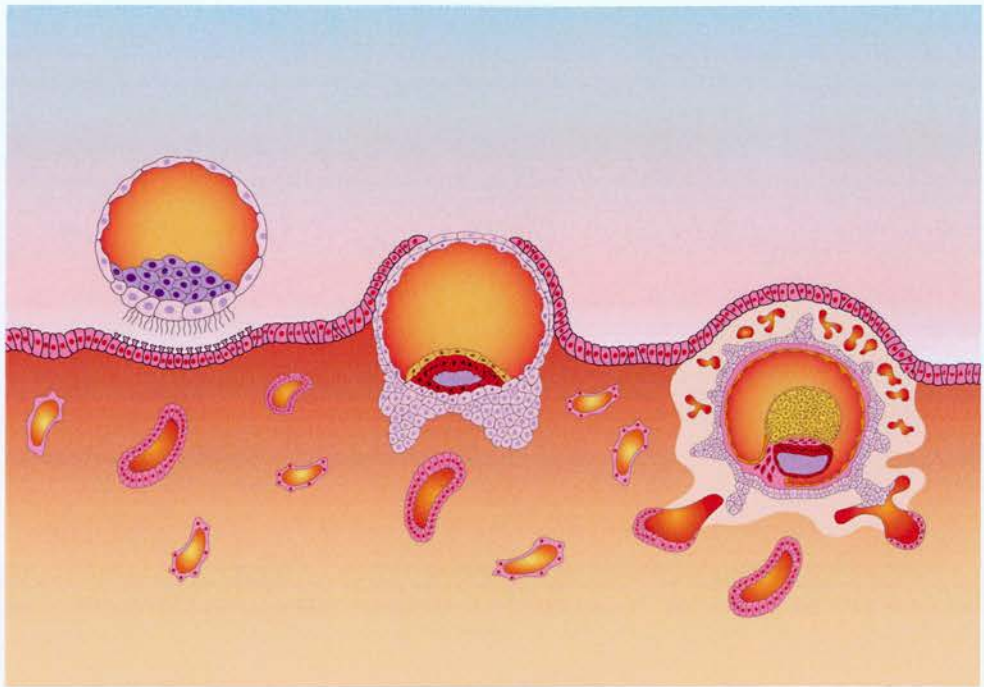

Figure 16 Schematic depiction of the process of implantation. Left of picture: The blastocyst in the pre-implantation stage triggering embryo-maternal cross-talk. Middle of picture: Nine to ten days post-conception, showing blastocyst invasion, to which HhCG has been suggested to contribute. Right of picture: Eleven to fourteen days post-conception, by which time the biological effect of hCG is fully functional. [Figure adapted from Reference 144.]

During the normal menstrual cycle, the uterine mucosa undergoes changes that reach maximum during the luteal phase, making it receptive for implantation when embryonic signals are present. Although mechanisms of implantation vary somewhat among species, *in vivo* and *in vitro* studies in animals (particularly baboons) can helpfully contribute to elucidation of the complex process of implantation in humans. In baboons, (as in humans), uterine receptivity may be divided into distinct phases [139, 145, 146]. Phase I is regulated by oestrogens (E) and progesterone (P) and occurs between post-ovulation Days 8 and 10 of the normal menstrual cycle. In this phase the uterine lining is neutral toward the implanting blastocyst. The second phase of uterine receptivity is induced by blastocyst "signals" superimposed on the E/P-primed receptive endometrium.

Probably through actions on the endometrial receptors, hCG in the receptive phase causes functional and morphological changes in three major cell types of the endometrium - the luminal epithelium, glandular epithelium and stromal fibroblasts [139, 147, 148]. In the luminal epithelium, hCG stimulates the plaque reaction (i.e. hypertrophy, hyperplasia and differentiation of the surface epithelium) possibly by infiltration of pro-inflammatory cells into the uterus. hCG also causes glandular

transformation and stromal cell differentiation by increasing transcriptional and post-translational modulation of glycodelin. *In vitro* studies show glycodelin A to be a paracrine modulator of hCG synthesis as it induces hCG secretion in culture media containing first and third trimester trophoblast cells. [149] It also regulates the uterine immune responsiveness by suppressing decidual thymidine uptake. This decreases synthesis of IL1 and IL2 and expression of the IL2 receptor by mitogen-stimulated cells, inhibits the cytotoxic activity of natural killer (NK) cells and suppresses both allogenic mixed lymphocyte reaction and responsiveness to phytohaemagglutinin [149].

A further action of hCG on human endometrium has been demonstrated in novel experiments in which urinary hCG was infused directly into the uterine cavity of women during the luteal phase using an intrauterine microdialysis device and changes in concentrations of various cytokines and growth factors in the uterus were determined [150, 151]. Vascular endothelial growth factor (VEGF) levels increased, suggesting direct involvement of hCG in the endometrial angiogenesis required for implantation. Concentrations of leukemia inhibitory factor were also significantly stimulated. This cytokine is produced by natural killer lymphocytes that interact with the invading trophoblast and has been shown to activate both urokinase plasminogen activator and gelatinase, enzymes which have crucial roles in trophoblast invasion [147]. Levels of MMP-9, an enzyme involved in tissue invasion during implantation, were also significantly increased.

In addition to these stimulatory effects, hCG had inhibitory effects on intrauterine levels of insulin-like growth factor binding protein-1 (IGFBP-1) and macrophage colony-stimulating factor. Both these have been shown to restrict trophoblast invasion into the decidua, highlighting the paracrine role of hCG on endometrial functions essential for implantation [151, 152].

A prerequisite for such a direct effect of hCG on the endometrium is the presence of functional LH/hCG receptors. HCG receptor mRNA is expressed in the endometrium at the time of implantation [147, 153]. Studies using immunohistochemical and *in situ* hybridization techniques revealed a cycle-dependent appearance and distribution of hCG structures and mRNA. No immunoreactive cells were detectable during the proliferative phase. However, in the secretory phase of endometrium, hCGβ-like structures are found in glands as well as in stroma [147]. Thus hCG secreted by the

glandular epithelium during the progesterone-induced secretory phase may contribute to maintaining the corpus luteum, as well as supporting early pregnancy.

In relation to stromal fibroblast modulation, hCG enhances the action of α-smooth muscle actin. This is possibly in response to integrins on the stromal cell membranes which bind to secreted extracellular matrix proteins causing stromal cell proliferation and differentiation [139]. The addition of hCG to steroid-treated stromal cells in baboons has been shown to inhibit apoptosis and enhance differentiation, as reflected by IGFBP-1 expression and an increase in the transcription of the pro-actin gene, both of which promote decidualization of stromal cells [139].

1.10.3 Role of hCG in angiogenesis

The cytotrophoblasts situated at the tip of the anchoring villi in the intervillous space proliferate outwards from the underlying basement membrane to form cell columns from which cells migrate into the interstitial trophoblast cells and invade the maternal spiral arteries, thus establishing the uteroplacental circulation [144]. For normal intrauterine fetal development and survival, adequate nutrient supply is mandatory and disturbances in uterine blood supply are generally associated with higher perinatal morbidity and mortality e.g. due to preterm delivery, pre-eclampsia, or intrauterine growth restriction. The vasculature of the uterus and surrounding tissues undergoes adaptive changes to accommodate the rising needs of the growing fetus by increased vasodilation, vascular permeability, and neovascularization.

In general, the angiogenic process is initiated by growth factors such as basic fibroblast growth factor, vascular VEGF, and placental growth factor. hCG is thought to stimulate endometrial angiogenesis and growth and extend the implantation window through its actions on IGFBP-1 and VEGF, both of which are considered key regulators of neovascularization and vascular function [148, 151, 154]. Exposure of human granulosa cells to hCG stimulates the expression of VEGF mRNA [155, 156]. Furthermore, administration of hCG in women undergoing *in vitro* fertilization has been shown to increase urinary VEGF concentrations [156]. Incubation of uterine artery extracts with highly purified hCG not only resulted in a dose-dependent increase in cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), prostacyclin synthase, and vasodilatory eicosanoids such as 6-keto-prostaglandin-F1α, but also in a decrease in thromboxane-A2 synthase, and vasoconstrictors such as prostaglandin-E2, and thromboxane-B2.

There was also a significant decrease in the resistance index of uterine arteries 16 hours after the administration of 10,000 IU hCG for ovulation induction in women, indicating an increase in uterine blood flow. The angiogenic response is possibly induced through activation of the protein kinase C-signaling pathway (PKC), which plays an important role in new capillary formation and survival phases of angiogenesis in different systems. PKC inhibitors decrease the angiogenic response in endothelial cells [148].

The direct angiogenic actions of hCG on fetomaternal vasculature are thought to be due to the presence of LH/hCG receptors in the endothelium and smooth muscle of uterine blood vessels. Indeed, the expression of LH/hCG receptor mRNA is shown to be higher in the intra-myometrial segment than in extra-myometrial arteries and is higher in endothelial cells than in the smooth muscle of intra-myometrial arteries [157]. The classical signal transduction pathway induced by hCG (i.e. cAMP-dependent G-protein signal transduction in gonadal cells) was not detected in a baboon endometrial epithelial cell line. Instead, an alternative PKA-dependent pathway involving activation of extracellular signal-regulated protein kinases 1 and 2 was proposed to bring about up-regulation of COX-2 expression and prostaglandin E2 production [158, 159].

1.10.4 Role of hCG in late pregnancy

There is increasing evidence that hCG also plays a role in maintaining late pregnancy through the hCG/LH receptors in uterine myometrial smooth muscle cells [160]. hCG inhibits myometrial contractions by down-regulating gap junctions and decreasing intracellular Ca^{2+} levels [161, 162]. Gap-junctions allow coordinated myometrial contractions in labor, their numbers peaking at the time of delivery and then decreasing thereafter. HCG may act via its receptors to enhance myometrial quiescence by down-regulating the myometrial-gap junctions and inhibiting contractions [163]. A direct effect of hCG on gap junction protein may also be exerted through the connexin protein units (CX) of gap junctions.

Using human myometrial samples obtained during caesarean sections, CX-43 protein levels have been shown to be down-regulated with increased duration of exposure to hCG, probably through protein kinase A signaling [161]. HCG also reversed the oxytocin-induced increase in CX-43 protein levels [161]. Myometrial LH/hCG receptor levels are not constant in the pregnant myometrium, as both the receptor protein and its mRNA level are lower during labour, both in term and preterm pregnancy [164].

Down-regulation of the LH/hCG receptor during labour may be a prerequisite for transition of the uterus from a quiescent state to the active stage of labour [163].

HCG can directly act on human myometrial smooth muscle cells, through the protein kinase A signaling system, to increase both their number and their size in a subpopulation of cells. This suggests a possible role in the expansion of the uterus in order to accommodate the growing foetus [165]. The angiogenic vasodilatory role of hCG mentioned earlier is not only critical for implantation but also may be essential for facilitating nutrient delivery and waste removal during later pregnancy. In a study using pregnant mice in which preterm labor was induced using prostaglandin F2α, a single high dose injection of hCG not only resulted in inhibition of preterm labor but also prolonged pregnancy beyond term [166]. A similar tocolytic effect of hCG was obtained when hCG was administered to a small cohort of women with established preterm contractions. [167] hCG was shown to suppress uterine contractions and delay labor without side-effects. These findings collectively suggest a uterotropic and quiescence- inducing role for hCG which could be further investigated to develop therapies for preventing or inhibiting preterm delivery.

1.10.5 Other roles of hCG

The role of hCG in ovarian development and differentiation is unclear. However hCG induces secretion of testosterone by fetal testicular Leydig cells, which is essential for the development of internal and external male sexual organs prior to the onset of pituitary LH secretion. This is supported by the following observations: 1) peak fetal serum testosterone levels coincide with peak maternal hCG concentrations, 2) testicular tissues from 14-16 week fetuses produce testosterone and cAMP when incubated with hCG, 3) the fetal pituitary contains an insufficient amount of LH at this time to account for fetal testicular testosterone production, and 4) the differentiation of Wolffian duct structures takes place only during the period of testosterone secretion by Leydig cells [109].

HCG has also been reported to have immunosuppressive properties and to induce immunological tolerance of the maternal immune system to the trophoblast and growing fetus [148, 168]. *In vitro* studies demonstrate that the secretion of several cytokines from peripheral blood mononuclear cells (PBMC) derived from women in early pregnancy is modified by hCG stimulation. hCG inhibited IL-2 production and increased IL-1β, IL-6, and TNF-α production, and also significantly enhanced

interferon γ production when calcium ionophores were used as inducers. Additionally, hCG diminished IL-2 secretion and enhanced release of soluble IL-2 receptor from PBMC. Basal production of IL-8 by PBMC (along with other cytokines) is increased very early (4-5 weeks) in pregnancy, after which the levels return to those produced by the PBMC of non-pregnant women. This, coupled with the finding that hCG activates monocyte production of IL-8 via a different pathway from the LH/CG receptor system [148, 168], possibly suggests a role for HhCG in regulating maternal immune response and preventing immuno-rejection of the developing fetus.

Because of its structural similarity to TSH, hCG can at high concentrations bind to TSH receptors causing excessive stimulation of the thyroid gland hCG, and contributes to the increased maternal thyroxine levels sometimes observed in the first trimester of pregnancy. In normal pregnancy, serum TSH levels fall when hCG peaks and are a mirror image of hCG levels. Free triiodothyronine (T3) and thyroxine (T4) levels are significantly elevated at this time, and this may be one cause of hyperemesis during early pregnancy. This is supported by the finding of thyroid hyperstimulation in cases of molar and multiple pregnancies, which are associated with higher levels of hCG [169]. Other roles of hCG include stimulation of dehydroepiandrosterone production by fetal adrenal glands and stimulation of decidual prolactin secretion. HCG also promotes placental steroidogenesis (by stimulating the conversion of cholesterol to pregnenolone and progesterone), placental aromatisation, and hydroxylation of placental steroids, although the precise mechanisms remain uncertain [109, 170].

1.11 The role of HhCG in pregnancy and implantation

As described earlier, the final phase of uterine receptivity is initiated after blastocyst attachment and implantation, ending in formation of the human haemochorial placenta, in which blood from the maternal circulation constantly bathes the fetal chorionic villi. Phenotypically, trophoblast cells are considered to be the physiological counterparts of invasive malignant tumors but have a controlled program of terminal trophoblast differentiation. It is unclear whether trophoblast cells utilize the same molecular growth mechanisms as tumors or whether they also have migratory and invasive capabilities. However studies have shown that hCG, while regulating its own biosynthesis, may simultaneously induce multiple signals that control trophoblast proliferation, in part by regulating the expression of key components required for

progression through the cell cycle, and in part by controlling protease activity. HCG is able to modulate parameters of endometrial differentiation such as IGFBP-1, and influence the invasive pathway by interfering with the production of MMP activators and their tissue inhibitors [141, 150, 155, 168, 171-174]. Thus hCG is one factor in the functional differentiation of human cytotrophoblast cells which regulates their proliferative potential.

HhCG is exclusively produced by the invasive cytotrophoblast cells at the time of implantation during pregnancy and accounts for the major proportion of total hCG forms produced during the period of trophoblast invasion [66, 67, 69, 70, 175]. It is also produced in high concentrations in choriocarcinoma and testicular germ cell tumors [72, 77, 176]. The immunoreactivity secreted into the conditioned medium of JAR choriocarcinoma cells is wholly due to HhCG [49]. Studies by Lei *et al.* showed that JAR cells are invasive in matrigel basement membrane inserts *in vitro* as well as when transplanted into athymic nude mice in vivo [73]. When JAR cells are treated with hCGα antisense cDNA, production of HhCG is blocked and invasion into the matrigel membrane *in vitro* and tumorigenesis in athymic nude mice *in vivo* is also compromised. These data suggest that HhCG has a critical role in trophoblast cell invasion.

HhCG has been shown to lack the biological action of hCG in maintaining the production of progesterone due to its poor binding capacity with the LH/hCG receptor, and shows only 4% of the steroidogenic action of hCG [126]. No role during implantation has as yet been confirmed for HhCG. However due to its cystine-knot configuration, the molecule may exert some of the cytokine-like action mentioned above during early pregnancy [23, 37]. As hCG has shown to down-regulate Fas, Fas ligand, Bax and p53, HhCG may be involved in growth and invasion of the trophoblast during implantation (or tumor development) by similar anti-apoptotic activity. It has been suggested it might exert such action by acting as an autocrine antagonist on the TGFβ receptor [177, 178].

In vivo and *in vitro* studies have shown that HhCG promotes formation of cytotrophoblasts and choriocarcinoma cell growth and invasion. Using a matrigel invasion model, addition of HhCG, but not hCG, has been shown to promote growth and invasion of pregnancy membranes by cytotrophoblast and choriocarcinoma cells in culture. In a further experiment, when JEG3 choriocarcinoma cells were

transplanted into nude mice, growth and progression of newly formed tumors could be rapidly blocked using mouse MAbs raised against HhCG [72, 74].

There is direct evidence of the invasion-promoter action of choriocarcinoma hCG from a study by Hamada et al [179]. Transfection of the anti-sense hCGß gene into JAR choriocarcinoma cells not only suppressed the expression of hCGß mRNA expression and hCGß protein synthesis, but also resulted in significant inhibition of cell proliferation by significant increasing the apoptotic rate of cells [179]. Indeed, an earlier study by Lei et al. demonstrated that the tumorigenic effects of choriocarcinoma hCG did not involve increased cell proliferation, but rather decreased apoptosis leading to increased invasion of JAR cells in nude mice [73]. It was suggested that this action was brought about through the cytokine-like action of choriocarcinoma hCG [73]. Hamada et al. concluded that the tumorigenic activity of the hormone involved its action on the LH/hCG receptor. [179]

It remains to be determined whether HhCG exerts its action through LH/hCG receptors or, due to the cystine knot configuration, to a cytokine-like action on TGFβ receptors that trigger apoptosis [70-72, 74, 180]. Together with the finding of unduly low levels of HhCG this suggests that insufficient production of HhCG itself may be the cause of ineffective implantation that leads to pregnancy failures [66, 67, 70, 81].

Binding of hCG to its receptor generates a signal transduction pathway, producing a classical response. This is characterized by an increase in cAMP and consequent activation of protein kinase A (PKA) upon activation of the adenylyl cyclase (AC) pathway and an increase in the intracellular calcium through inositol triphosphate (IP3)/ phospholipase A2 (PLA A2) pathway. However HhCG is unlikely to be involved in such a conventional signal transduction pathway because it does not bind to the hCG/LH receptor. Experiments using endometrial epithelial cell lines from human and baboon have shown that hCG does not activate the AC-cAMP-PKA pathway but that it can rapidly induce phosphorylation of the extracellular signal-regulated kinase (ERK 1/2) in a PKA independent manner leading to an increase in COX-2 mRNA and PGE2 production [158]. This suggests the existence of an alternate spliced isoform of the hCG/LH receptor or presence of a different form of hCG that can activate alternative signal transduction pathway. While functional hCG/LH-receptors are expressed on both cytotrophoblasts and syncytiotrophoblasts, syncytiotrophoblasts express the highest level of hCG/LH receptors [160]. The fact that HhCG is dominant around the time of implantation and in the following 3 weeks

suggests that HhCG may play a key role in embryo-maternal cross-talk, long before hCG is measurable in maternal blood. If so, whether such action is mediated through the action on receptor-tyrosine kinases or through increased expression of specific adhesion receptors and extracellular matrix-degrading proteases such as collagenases and plasminogen activators, has yet to be elucidated.

Based on available data, hCG can exert a range of local and systemic actions, in and outside the embryo-endometrial microenvironment, and during different stages, indicating its important juxtacrine, endocrine and autocrine functions during early pregnancy. The exact contribution of HhCG in exerting similar is yet to be elucidated.

1.12 Physiology of pregnancy complications

Pregnancy complications may be associated with genetic, nutritional, immunologic, and infectious factors that lead to pathological changes such as abnormal placentation, oxidative stress, and endothelial dysfunction (Figure 17), implantation and early placental development, and under the influence of relatively hypoxic conditions, cytotrophoblasts proliferate and invade rather than differentiate, causing a rapid increase in placental mass. While still under a state of physiological hypoxia, cytotrophoblasts invade the uterine wall and attaches to maternal vessels. By 10-12 weeks of human pregnancy, blood flow to the intervillous space is established. As the endovascular component of cytotrophoblast invasion progresses, the cells migrate along the lumina of the spiral arterioles, replacing the maternal endothelial lining.

In normal pregnancy, oxygen tension influences both the differentiation process that leads to the invasion of cytotrophoblast into the uterus and the degree of oxidative stress that in turn modulates placental hormone synthesis [181-183]. During normal The process of placental remodeling of uterine arterioles, which involves the decidual and inner third of the myometrial portions of these vessels, causes the diameter of the arterioles to expand. This converts their muscular walls into low-resistance channels, which can accommodate the demand for increasing blood flow required to support fetal growth. Failure of endovascular invasion and inability of the cytotrophoblast to invade to the appropriate depth has been linked with spontaneous miscarriage, pre-eclampsia, and fetal growth retardation (FGR).

It has been shown that premature onset of the maternal placental circulation reduces trophoblast invasion and results in incomplete plugging of the maternal spiral arteries. This may be due to a primary defect in the trophoblast lineage (e.g. a zygotic

chromosomal abnormality) or to adverse secondary changes in the endometrium (eg, infection), leading to stress in the syncytiotrophoblast and some loss of cytotrophoblast [184].

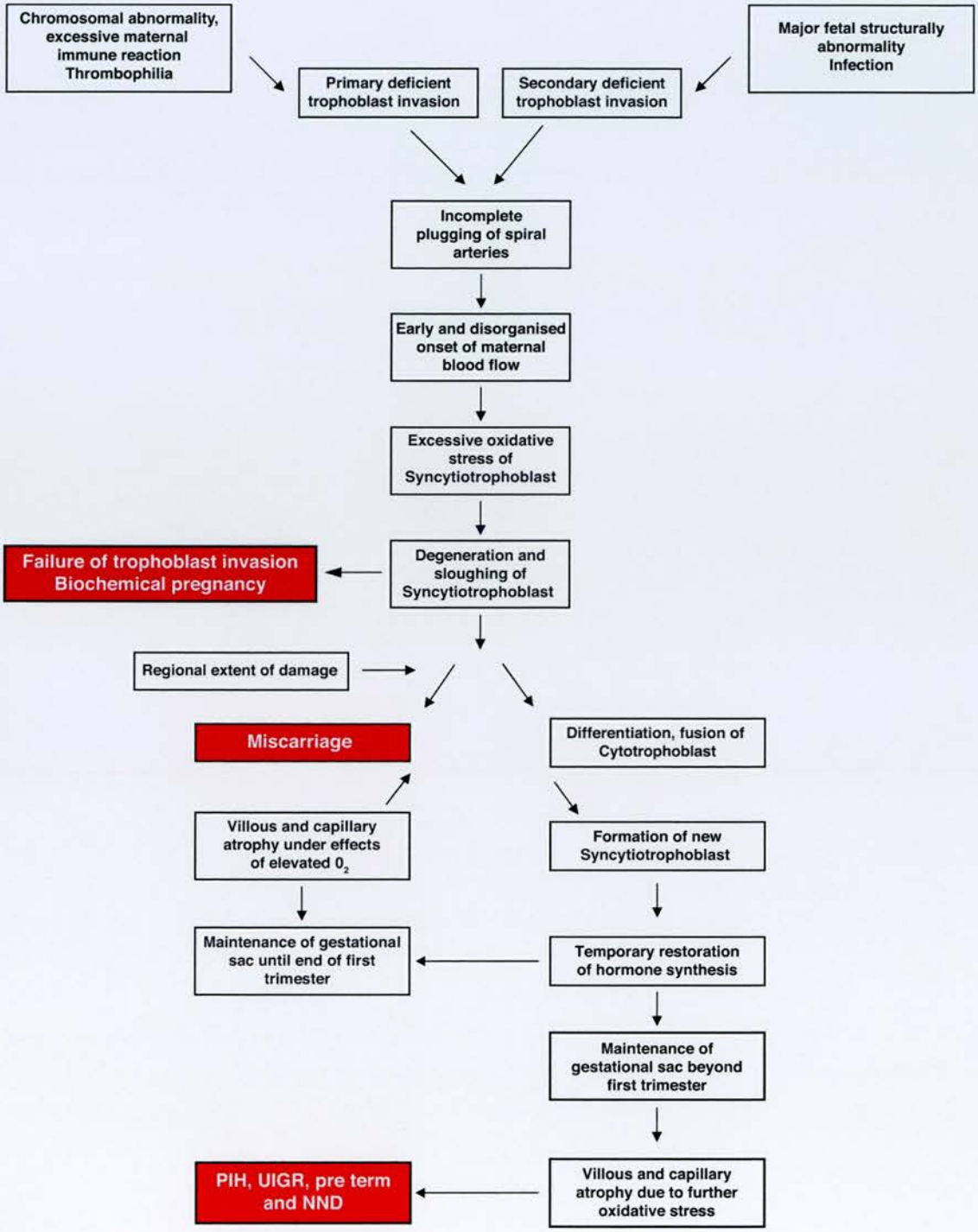

Figure 17 Schematic diagram showing proposed underlying mechanisms and common pathophysiology of some common pregnancy complications. [Figure adapted from that in Reference 181.]

Hence, it is speculated that if syncytial degeneration is extensive, the pregnancy may be lost as a spontaneous miscarriage. However, if the insult is less severe, sufficient hormonal activity may be regained through syncytial regeneration from the differentiation of surviving cytotrophoblast, allowing the pregnancy to continue as a missed miscarriage. A similar mechanism may be involved in the appearance of later complications, in which the pregnancy may continue following temporary restoration of hormone synthesis and containment of initial oxidative-stress related damages. [184]. However, the presence of additional stressors or deficiencies in antioxidant defences later in gestation may increase the likelihood of placental dysfunction and oxidative tress-related damages, leading to complications such as pre-eclampsia, FGR, or fetal death (Figure 17).

Obstetric complications associated with inadequate trophoblastic invasion during the first trimester may be associated with low placental hormone levels. There are three contributory mechanisms, existing alone or in combination with one another, that may lead to elevations in the maternal circulatory placental hormone and protein levels. One mechanism is the "reactive hyperplasia of cytotrophoblastic cells" as a compensatory response to reduced blood supply, leading to increased production of placental hormones in an environment of low oxygen tension. Indeed, the degree of oxidative stress has been proven to modulate placental hormone synthesis [181-183]. Oxidative stress generated by low concentrations of hydrogen peroxide (H_2O_2) enhances cytotrophoblast hCG secretion, whereas higher concentrations of H_2O_2 reduce hCG secretion in a dose-dependent manner [181, 185]. Pathological placental changes and an abnormal trophoblastic secretory response are other mechanisms through which placental hormone immunoreactivity in the maternal circulation may be increased. Morphological analyses provide convincing evidence of perturbed villous development in pregnancies with pre-eclampsia and FGR.

Aberrant features observed include increased cytotrophoblasts, increased syncytial knots, increased apoptosis, focal cellular necrosis in syncytiotrophoblasts and increased mitotic activity in syncytiotrophoblasts, accompanied by cellular proliferation and hyperplasia of cytotrophoblasts along with their differentiation into new syncytiotrophoblast tissue [186-190]. The natural steady state between cytotrophoblast and syncytiotrophoblast, whereby the syncytiotrophoblast is formed and replenished by the underlying cytotrophoblast, can be disrupted in late pregnancy complications. If the balance between the rates of cellular loss and replacement of

syncytiotrophoblasts changes to favor replacement, this may lead to high serum levels of hCG. Alternatively, hyperplasia of the cytotrophoblast without the formation of syncytiotrophoblast may lead to an increase in the levels of one hCG glycoform, a mechanism that may explain the increase of hCGα levels, which are produced in excess by the cytotrophoblast following the failure of implantation [187, 191].

As hCG synthesis requires both hCGα (produced by the cytotrophoblastic cells) and hCGβ (secreted by syncytiotrophoblastic cells), a growing population of cytotrophoblasts and excessive amounts of syncytiotrophoblasts derived from the cytotrophoblast, may lead to high total hCG levels in the maternal circulation. The third mechanism postulated is that alterations in the surface layer of the syncytiotrophoblast as a result of ischaemic damage associated with abnormal trophoblastic invasion lead to a rise of placental hormones as a consequence of their "increased leakage" into the maternal circulation [183, 192].

The complex mechanisms involved in pregnancy complications and the unpredictable placental response in the wake of such complications and the consequent production of heterogeneous hormones, all together make screening for pregnancy complications extremely challenging. In general, the data in the literature about the usefulness of hCG in the prediction of pregnancy complications are discordant. There are many inconsistencies between study results, which will be the focus of discussion in following chapters.

1.13 Measurement of hCG and related molecular forms

Urine-based pregnancy tests date back to as early as 1350 BC when an ancient Egyptian papyrus described a test in which a woman who might be pregnant could urinate on wheat and barley seeds over the course of several days: "If the barley grows, it means a male child. If the wheat grows, it means a female child. If both do not grow, she will not bear at all." Testing of this theory in 1963 found that 70 percent of the time, the urine of pregnant women did promote growth, while the urine of non-pregnant women and men did not. In the literature, this is perhaps the first test to detect a unique substance in the urine of pregnant women. [193]

Clinically useful bioassays were introduced by Ascheim and Zondek (1927) [9] Friedman (1931) [194], Hogben (1939) [195] and others. These tests could confirm pregnancy approximately two months after a missed period but took days to complete and required the sacrifice of an animal. They were replaced in 1960 by a

haemagglutination inhibition test [196]. Purified hCG was mixed with the urine sample and antibodies directed against hCG. In a positive pregnancy test, the red cells agglutinated. The test took only two hours to perform and was less expensive than bioassay. However it was relatively insensitive, with a detection limit of 500 U/L of hCG, and cross-reacted with LH. The development of radioimmunoassays allowed detection of hCG at much lower concentrations [193].

Currently available assays for hCG can be broadly classified as methods (measuring predominantly hCG) or total methods (measuring hCG and hCGβ and sometimes other molecular forms as well). Specific assays are also available for hCGα, hCGβ, and hCGβcf (Figure 18).[197]

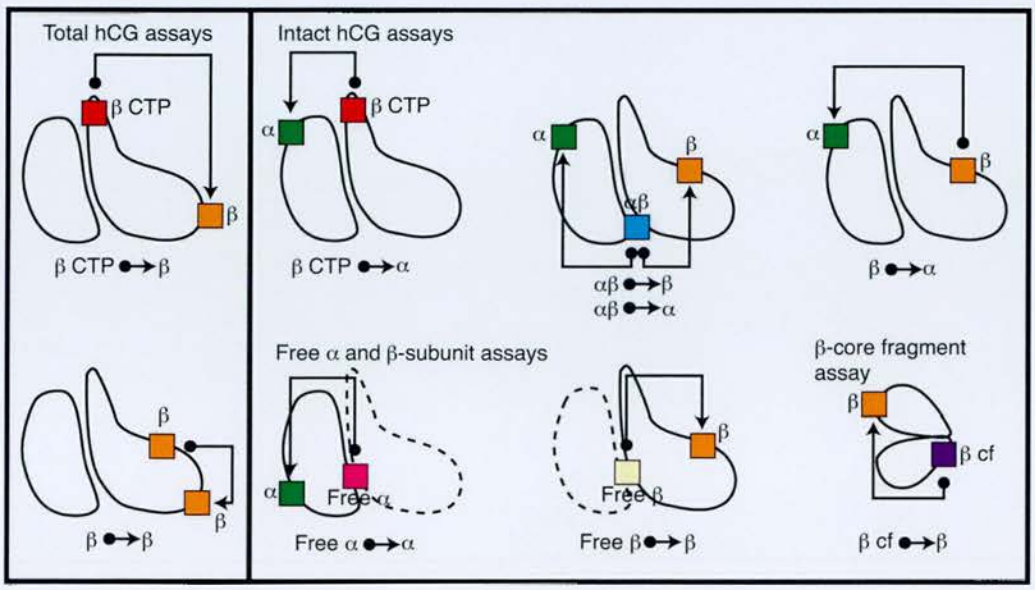

Figure 18 Schematic representation of antibody combinations enabling measurement of hCG and related molecules. [Adapted from Reference 197.]

1.13.1 General principles

Most two-site immunoassays, whether qualitative or quantitative, are developed using two carefully selected MAbs directed against different antigenic determinants on the molecule to be measured. The capture antibody is usually bound to a solid phase while the detection antibody remains in solution and is labelled with a signal molecule. In the presence of the antigen to be measured, a "sandwich" complex is formed. This is separated from the rest of the reaction mixture and the amount of label bound to the solid phase is then determined by reference to a standard curve (Figure 19).

Figure 19 Schematic representation of the principle of two-site non-competitive immunoassay. The amount of analyte (■) in an unknown sample is determined by comparing the amount of labelled MAb (⊢•) bound to solid-phased antibody (○⊣) with a standard curve prepared using known amounts of antigen.

A variety of solid phase media can be used in immunoassay, including microtitre wells, beads, and gels. The label is usually enzymatic, chemiluminescent or fluorometric. Two-site immunoassays have largely replaced the previous competitive one-site radioimmunoassays because two-site immunoassays are faster, have wider working ranges and do not use radioactive labels. The specificity of an immunoassay is determined by the characteristics of the antibodies employed while its performance is strongly influenced by assay design and calibration.

1.13.2 Quantitative immunoassays for hCG and hCGβ

Currently few quantitative hCG assays measure hCG alone, and most are designed to measure both hCG and hCGβ. As they are calibrated against hCG, they may not recognize hCG and hCGβ equally [47, 198]. For pregnancy applications measurement of hCG is considered sufficient but for diagnosis and management of trophoblastic disease and germ cell tumours, assays that also recognize hCGβ are mandatory. Assays with broad specificities similar to those achievable using polyclonal antibody radioimmunoassays are generally regarded as desirable for these applications.

1.13.3 Quantitative immunoassays for HhCG

HhCG would be an ideal molecule for early pregnancy detection, as it is produced by the invasive trophoblasts at the time of implantation and during the weeks following this [66, 67, 69, 129]. Several immunoassays have therefore been developed for the specific detection of HhCG using MAb B152 in combination with an anti-hCGβ tracer [49, 69, 129, 199].

Most early studies of HhCG measurements in pregnancy, Down syndrome-affected pregnancy and gestational trophoblastic disease were performed using manual enzyme-linked immunosorbent (ELISA) and immunoradiometric (IRMA) assays [66, 67, 71, 200-202]. Later studies employed an automated immunochemiluminometric assay

(ICMA) [Nichols Institute Diagnostics Advantage® platform (San Clemente, CA, USA)] which is no longer commercially available. While the early manual assays had ~ 60% cross-reactivity with HhCGβ and detected all charge molecular forms of HhCG, the automated assay detected only about 25% of HhCGβ and failed to detect some of the charge variants. These differences may account for some of the variations HhCG results in the literature. Until an optimal assay is developed and an International Standard is available, concentrations of HhCG obtained using different methods will continue to vary.

1.13.4 Quantitative immunoassays for hCGβcf

HCGβcf has been investigated as a potential cancer marker in urine but large variations in its day-to-day levels severely limit its clinical utility [95, 203-205]. A similarly fluctuating pattern in day-to-day urinary excretion of hCG has been revealed during early pregnancy [113].

Even though blood is generally preferred to urine for hCG assays, urinary assays are clearly advantageous for point of care or home testing as well as for some epidemiological studies e.g. when investigating the impact of environmental toxins on fertility.

1.13.5 Quantitative immunoassays for hCGn

Nicked hCG can be detected in small amounts in the serum and urine of pregnant women from 7 weeks of gestation but is virtually undetectable earlier. Specific nicked hCG assays have been developed using C5, a choriocarcinoma-derived hCG isoform with 100% nicking in its β-subunit [18, 49]. These assays provide valuable research tools to study the role of hCGn in pregnancy, but a clinical application for specific determination of hCGn has not yet been demonstrated.

1.13.6 Qualitative immunoassays for hCG

While the assay principle is the same as for quantitative immunoassays, qualitative hCG assays are marketed for use by non-laboratory personnel, who are – often nurses in hospital wards (point of care testing) or women who suspect they are pregnant ("over-the-counter" tests) – and do not require complex laboratory equipment.

Urine is placed on a single use assay cassette and is absorbed through changing densities of nitrocellulose, concentrating the antigens into a narrow band. The urine

mixes with a dye-labelled anti-hCG tracer antibody, stored in a sponge at the proximal end of the device. The "hCG + antibody dye" complex formed migrates through the nitrocellulose, eventually reacting with a stationary band of an immobilized secondary capture antibody directed against a different part of the hCG molecule. The resulting complex generates a coloured test line (Figure 20). Coloured lines indicate a positive test result (usually at a concentration of >20 U/L). Remaining antibody dye migrates further through the device to a second stationary control line. Excess antibody dye will bind the immobilized hCG, forming a colour and confirming that the test has worked correctly.

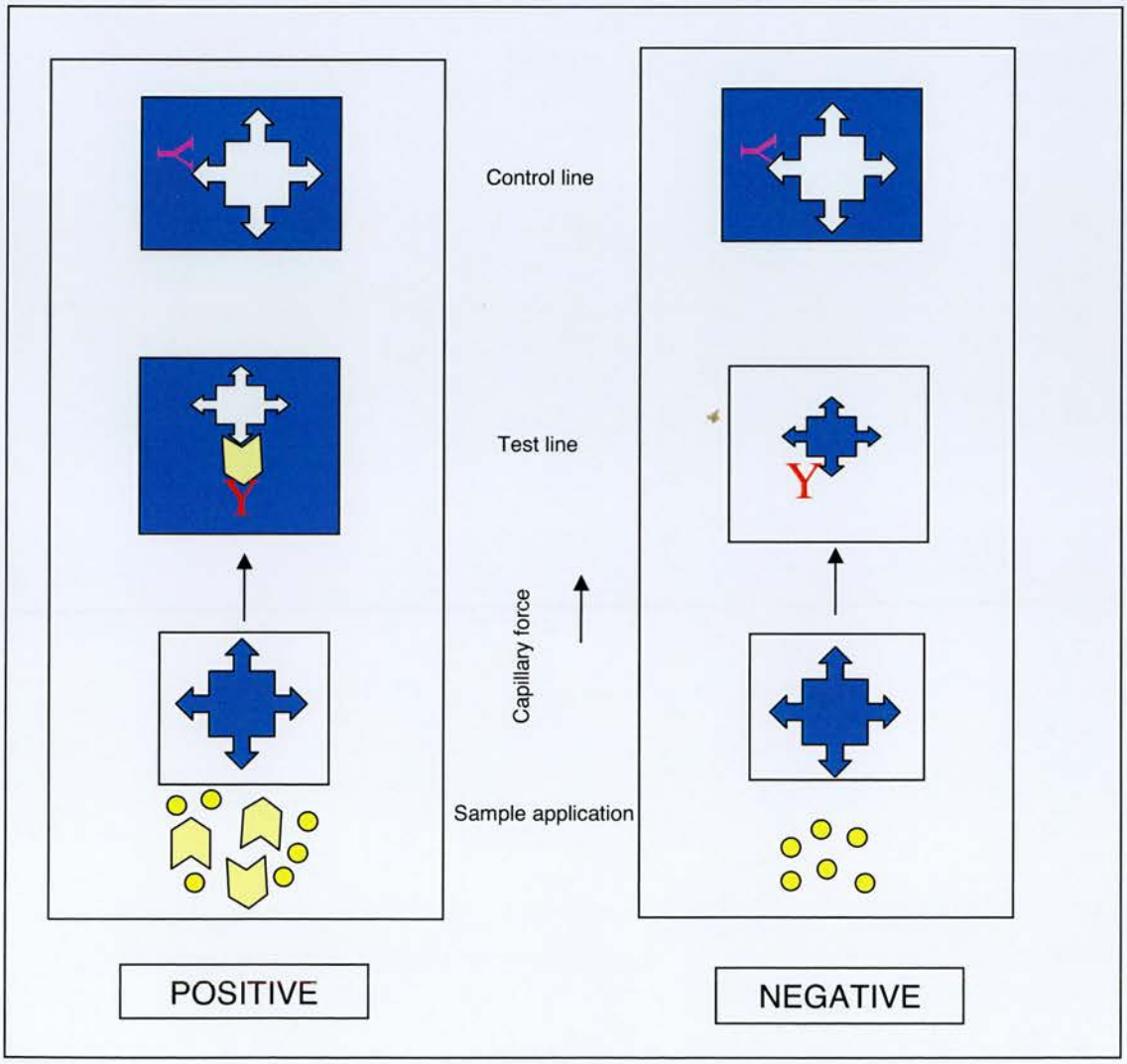

Figure 20 Schematic diagram of a qualitative test for hCG. Urine is applied onto a membrane and diffuses until it meets immobilized anti-hCG MAb coupled to a signal dye molecule. In the presence of hCG a complex is formed. This complex diffuses further down the membrane and subsequently reacts with a second anti-hCG MAb (Y), and the complex is deposited colour on the test band. Excess reaction mixture travels further into an area containing anti-species MAb (Y) with which colour will develop in the presence or absence of hCG in urine. This serves as the control line.

The desirable sensitivity for qualitative hCG tests is debatable [95]. As hCG may occur in serum and urine of non-pregnant women at concentrations of up to 10-15 U/L, a detection limit of around 25 U/L is likely to be optimal. Some experts suggest that highly sensitive home test kits are undesirable, as up to one third of all pregnancies detectable at implantation ("biochemical pregnancies") may fail very early [206]. Detection of these events is of uncertain benefit to women. Studies have shown that these pregnancies primarily produce hCG, and little or no HhCG [65, 67]. If this is correct, optimizing pregnancy tests so that they detect HhCG may help to distinguish clinical pregnancies from biochemical ones.

Literature reports suggest that measurement of HhCG may also be helpful in *in vitro* fertilization (IVF) programmes in which women are given hCG to promote maturation of follicles. Exogenous hCG persists for over two weeks, before endogenous production from a pregnancy (if successful) can be demonstrated. Specific qualitative or quantitative measurement of HhCG before exogenous hCG has cleared could help to reduce the anxiety of IVF patients who are eager to learn about their success or failure in achieving pregnancy.

1.14 Standardization of hCG assays

1.14.1 International Standards for hCG, hCGα and hCGβ

In 1938 the World Health Organization (WHO) established the 1st International Standard (IS) for hCG. The standard was prepared from pregnancy urine and was relatively crude, as was the 2nd IS which replaced it in 1964. The 3rd IS (75/537) was prepared from a more highly purified preparation, also from pregnancy urine, and used from 1978 to 1999. It was replaced by the current 4th IS (75/589), which was prepared from the same batch of material. HCG results are currently reported in International Units (IU) of hCG IS 75/589. International Reference Preparations (IRP) for hCGα (IRP 75/569) and hCGβ (IRP 75/551) were also established.

Ampoules of these standards [which can be obtained from the National Institute for Biological Standards and Control (NIBSC) (South Mims, UK)] have nominal mass contents assigned, allowing approximate calculations of relative reactivities (Table 3). However, these calculations require a number of assumptions to be made and they can only be considered as approximate. Approximately 9% of hCG IS 75/589 is nicked in

the region of β-subunit residues 43-48 (Table 3). This is undesirable as variable recognition of hCGn has been suggested to contribute to the differences in clinical hCG results obtained in different immunoassays. [83].

hCG-related species	International Standards (IS)	Source	Purity	Value assignment
hCG	IS 75/589 (4^{th} IS)	Pooled pregnancy urine	Contaminated with 10-20% hCGn	Arbitrary units based on earlier hCG standards calibrated by bioassays
hCGα	IRP 75/569 (1^{st} IRP)	Prepared from urine by dissociation of hCG	<1% contamination with hCGβ	Based on mass. One IU corresponds to 1 µg.
hCGβ	IRP 75/551	Prepared from urine by dissociation of hCG	<1% contamination with hCGα	Based on mass: One IU corresponds to 1 µg

Table 3 Properties of the current IS for hCG, hCGα and hCGβ

1.14.2 International Reference Reagents (IRR) for hCG, hCGn, hCGα, hCGβ, hCGβn and hCGβcf

Highly purified new International Reference Reagents (IRR) for six important forms of hCG were established by the WHO in 2001 (Table 4), with ampoule contents provided in molar units [62]. This is desirable since immunoassays reflect molar concentrations of protein rather than bioactivity [207]. It also simplifies comparison of recognition of the molecular forms in different assays. In addition, HCG IRR99/688 contains no hCGn and negligible amount of free subunits (Tables 5A and B) [62]. This preparation is currently being considered as a candidate replacement for IS 75/589.

1.14.3 Standards for HhCG

As the first report of HhCG only appeared a year after preparation of the six hCG IRRs began, a standard for HhCG was not included in that project. Manufacturers produce their own standards for their assays, usually by partially purifying HhCG from culture fluid from JEG3 choriocarcinoma cell lines. Unlike hCG, for which starting material is available in abundance from pregnancy urine in abundance or by recombinant technology, HhCG is present in urine at relatively low concentrations and peaks in very early pregnancy (3-6 weeks), making efficient extraction difficult. It

cannot currently be prepared by recombinant technology and other sources of high HhCG levels in urine, (e.g. choriocarcinoma) are unsuitable, as the HhCG from these sources is relatively unstable due to high levels of nicking. Establishing an IS for HhCG remains a priority.

Property	4th hCG IS 75/589	1st hCG RR 99/688
Biological activity	9286 IU/mg	1.1-1.5 times more potent/mg
Polypeptide chain structures	Approximately 10% nicked	Minimal
Amino Acid analysis	Agrees with structure	Agrees with structure
Sialic acid content % by weight	10.6	11.1
hCG related contaminants	Significant: hCGn some hCGβ + hCGβ cf	No significant contaminants
Protease activity	Present	Negligible

Table 4 Comparison of some of the properties of hCG IS 75/589 and hCG IRR 99/688.

Standard or relationship	Description	Calculation
hCG IS 75/537	Approximately 70 μg is equivalent to 650 U. Assuming molecular weight of 36.7 kDa.	I U ≈ [70 x 10^{-6} x 10^{12}] ÷ [36.7 x 650] picomoles ≈ 2.9 picomoles
hCGβ IRP 75/551	Approximately 70 μg is equivalent to 70 U. Assuming molecular weight of 22.2 kDa	I U ≈ [70 x 10^{-6} x 10^{9}] ÷ [22.2 x 70] picomoles ≈ 45 picomoles
hCGα IRP 75/569	Approximately 70 μg is equivalent to 70 U. Assuming molecular weight of 14.5 kDa	I U ≈ [70 x 10^{-6} x 10^{9}] ÷ [14.5 x 70] picomoles ≈ 69 picomoles
Mathematical relationship between units of hCG and hCGβ	**Required assumption:** hCG and hCGβ are recognized in the assay being considered on an equimolar basis	[1 U hCGβ] ÷ [1 U hCG] ≈ [45 ÷ 2.9] ≈ 15.5

Table 5A Relationships between International Units (IU) and molar units for the current IS for hCG, hCGα and hCGβ.

Preparation	Intact hCG %	HhCG	Nicked hCG	hCGβ	hCGα	hCGβcf
WHO 3rd IS/4th IS	100	15	9	0.8	1.4	0.8
99/688 (WHO 1st RR)	100	3	<0.1	0.9	1.0	0.2
hCG preparation P9	100	9	<0.1	<0.5	<0.1	2.3
CAP C15	100	0.9	<0.1	0.2	ND	<0.1

Table 5B Composition and homogeneity of purified individual hCG preparations and hCG standards [208]

1.15 Limitations of hCG tests

1.15.1 Interference from anti-reagent antibodies

False-positive hCG results may be obtained if interfering substances are present in a patient's serum. Such results are most often attributable to anti-reagent antibodies. In two-site (sandwich) immunoassays, a bridge may form between the capture and signal antibodies in the absence of antigen, as shown below, giving a false-positive (Figure 21a) or false-negative (Figure 21b) result.

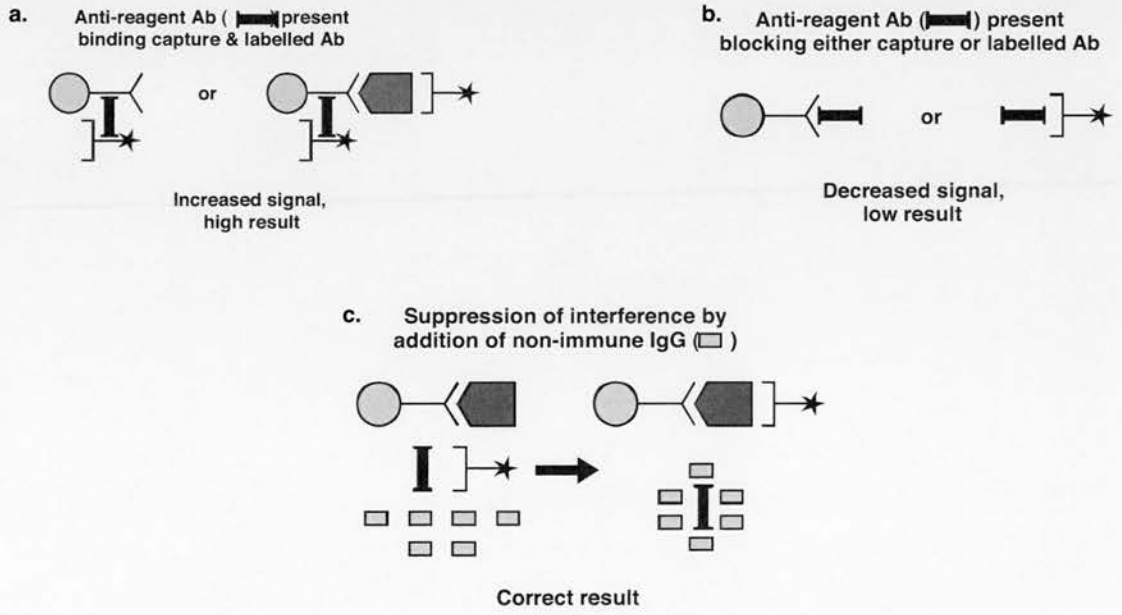

Figure 21 Schematic representation of anti-reagent antibody interference in a two-site non-competitive immunoassay. Such interference can result in falsely high (Figure 21a) or low (Figure 21b) results and its risk is reduced by addition of non-immune immunoglobulin G (IgG) (Figure 21c) [Analyte = ; labelled MAb = ; solid-phased antibody = ; anti-reagent antibody = ; non-immune IgG = .]

Interference from anti-reagent antibodies is most frequently caused by heterophilic or human anti-mouse antibodies (HAMA), which can bind the animal antibodies (e.g.

mouse) used in many hCG assays. Heterophilic antibodies may be induced by ingestion of cow's milk and other foods of animal origin or by direct contact with animals, and are reportedly found at low concentrations in 30-50% of healthy individuals [209]. Human anti-mouse antibodies may occur following injection of mouse antibodies for tumor imaging or therapy, or after administration of unconventional therapies [209,210]. Rheumatoid factors, which are IgM autoantibodies that are present in serum from some patients with rheumatoid disease, may also react with animal antibodies but are less likely to cause problems.

Strategies use to minimize these problems include the addition of blocking reagent to the reaction mixture (Figure 21c), or pretreatment of samples prior to assay with blocking agents such as polymerized IgG and polyclonal IgG. Blocking agents are not always effective since some sera contain high concentrations of heterophilic antibodies that cannot be neutralized [211]. Some antibody pairs appear to be more prone to producing false-positive results and are more difficult to remove [209,211].

1.15.2 Erroneous results due to high dose hooking

The 'hook effect' occurs when analyte is present in such high concentration that most binding sites on both capture and signal antibodies are occupied. This prevents the formation of the complex and leads to an erroneously low result (Figure 22). If undetected, the incorrectly low result reported can result in mismanagement of patients. This is a major potential limitation of one-step qualitative and quantitative serum and urine immunometric assays in which both capture and labelled antibody are added simultaneously. It is less likely to occur in two-step assays, where the analyte excess is washed away before tracer antibody is added [210]. The concentration at which high dose hooking may occur depends on the assay.

Assaying specimens at 10- and 100-fold dilutions, introducing a wash step prior to the addition of second antibody reduces the risk of errors due to the high dose hook or using kinetic rate measurements can all help to decrease the risk of failing to detect a hooked sample.

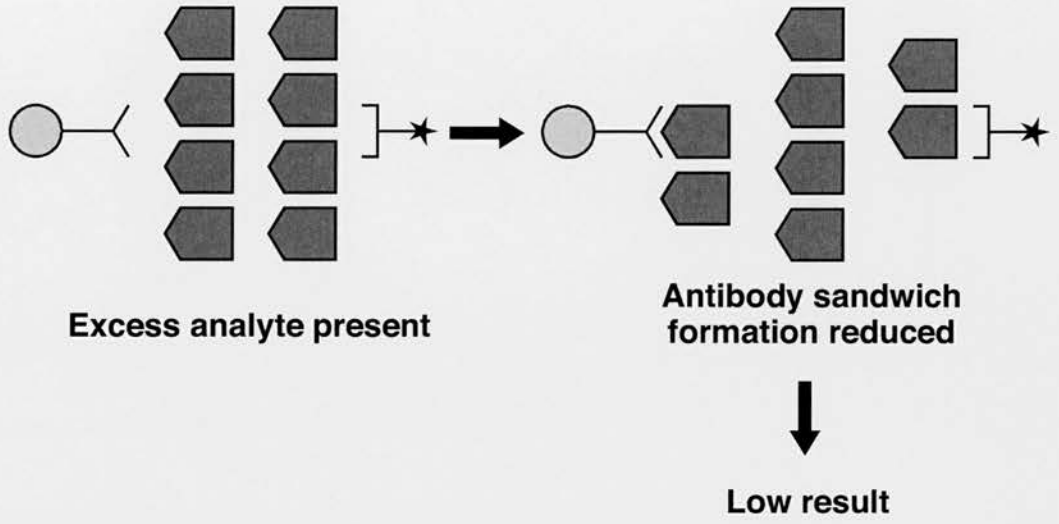

Figure 22 Schematic representation of the hook effect, which may cause falsely low results in the presence of very high concentrations of analyte. [Analyte =●; labelled MAb = ⊢• ; solid-phased antibody = ○⊣] See text for details.

1.15.3 Interference from complement and other factors

Various complement factors can react with antibodies giving different results depending on the isotype of the antibody used. In a sandwich assay, the complement which reacts with mouse IgG2 can cause low results, i.e. the effect is opposite to that of heterophilic antibodies, because complement reduces the binding capacity of the antibody. In contrast, complement will cause a false-positive result in an inhibition assay. Because of this, most assay manufacturers avoid using IgG2 antibodies in their systems. Other serum factors can also block the antigen-antibody reaction non-specifically. Lipids, haemoglobin, paraproteins and other serum constituents tend to be more of a problem in homogeneous assays than in two-site immunometric hCG assays.

1.15.4 Possible interference following administration of hCG

Anti-hCG antibodies produced by therapeutic use of urinary hCG will cause false-positive results in inhibition assays and false low results in sandwich assays. In earlier hCG assays, low false positive levels were noticed when serum LH was elevated (e.g. in menopausal patients) due to molecular similarities between LH and hCG. False-positives caused by such crossovers were generally in the 5-10 U/L range and would quickly be suppressed to less than 5 U/L with oral contraceptive treatment [134]. Intramuscular hCG injections at a dose of 10000 IU given to patients for ovulation induction in hormone-stimulated cycles can cause serum levels to peak to 200-300

U/L (500-1000 pmol/L) one day after injection, but levels decrease to <5 U/L within 8-12 days [95]. Athletes who use hCG to stimulate gonadal steroid production for doping purposes also may also present with elevated serum values [134].

1.16 hCG antibodies and epitope mapping

Improved comparability of results requires not only correct calibration of methods in terms of well-characterized IS but also us of antibodies with specificities most appropriate for particular clinical applications. HCGβ, HhCG, hCGn and hCGβn are important molecular forms of hCG found in Down syndrome pregnancy, gestational trophoblastic diseases, and some other cancers [207, 212] and it is important that immunoassays recognize these molecular forms as required.

Epitope mapping workshops have been organized by the International Society of Oncology and Biomarkers (ISOBM) with the aim of defining appropriate antibody combinations for different clinical applications. In a collaborative multicentre study, the ISOBM TD-7 hCG workshop characterized the molecular epitope structure and specificities of a panel of antibodies from various manufacturers [198]. Using competitive and sandwich RIA, ELISA and Western blotting, 27 MAbs were characterized using a variety of preparations including the 1st WHO IRR and LH and LHβ subunits from various species (Figure 23) [198]. Three-dimensional molecular epitope assignment was achieved by comparing immunoreactivity of the 27 ISOBM antibodies with those of an independent panel of 18 reference antibodies.

Five epitopes are located on hCGα (α1-α5), and seven on the beta-subunit of hCG. The β8 and β9 epitopes positioned on the CTP are completely specific for hCG and hCGβ, so many commercial assays utilize antibodies that recognize these two epitopes. Four epitopes (C_1 - C_4) are specific for heterodimeric hCG. Two of these (C_1 and C_2) are lost in nicked hCG. Epitopes α6 and α7 are specific for hCGα while β6 and β7 epitopes are specific for hCGβ. Epitopes recognized by hCGβ MAbs are located on the first and third loops protruding from the cystine knot of hCGβ (β2-β6, hCGβ 20–25 and 68–77), centered around the knot itself (β1), or on hCGβCTP (β8, hCGβ 141–144; β9, hCGβ 113–116) (Figure 23) [198].

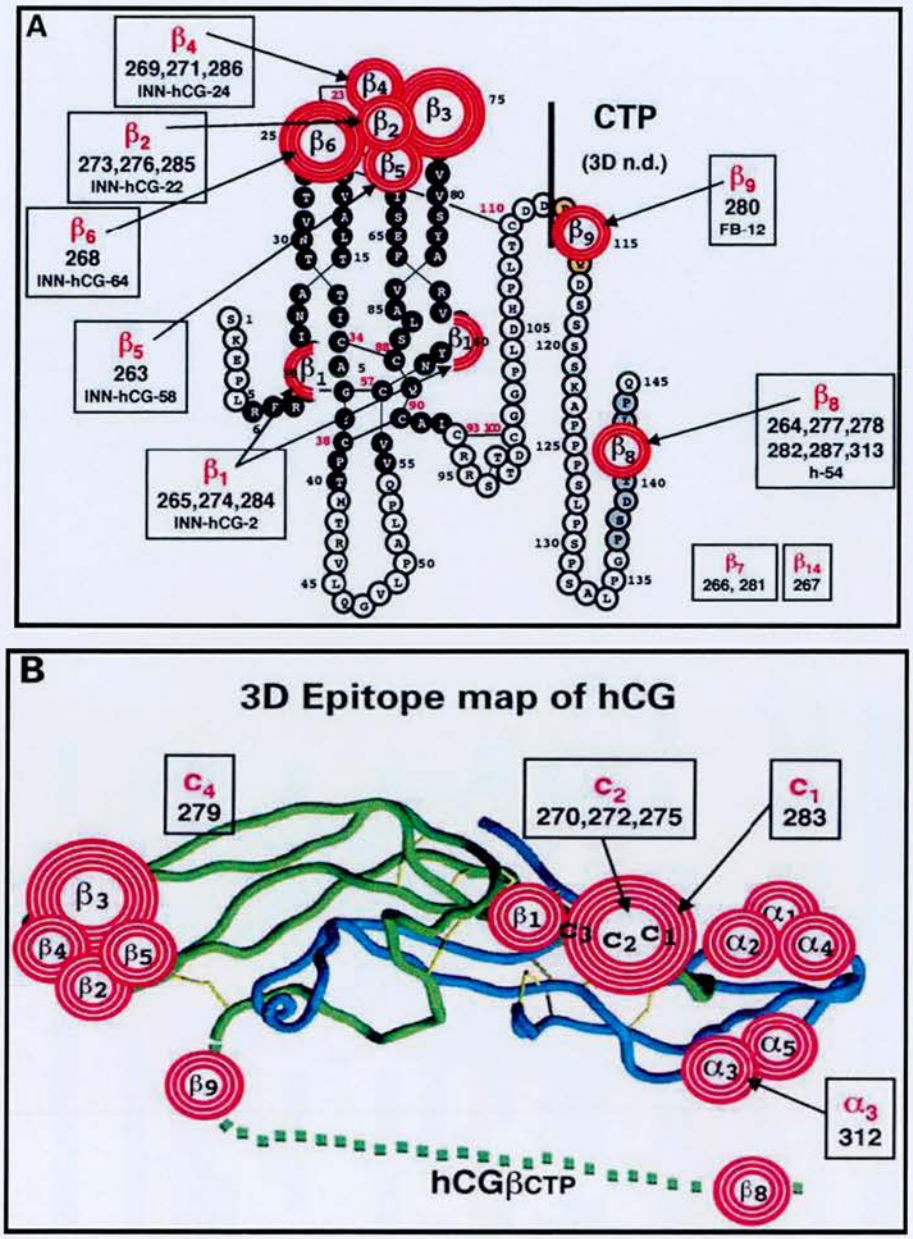

Figure 23 Schematic diagram of ISOBM TD-7 workshop results, showing assignment of epitopes of (A) hCGß/hCGßcf/ hCGßCTP and (B) hCG based on their three-dimensional structure. hCGßCTP is represented as dotted lines. [Figure reproduced from Reference 198 and used with permission.]

Use of assays recognizing a broad spectrum of hCG/hCGβ molecular forms is generally recommended for most diagnostic purposes. Low cross-reactivity with related glycoprotein hormones such as LH is also mandatory. These criteria can be met by combinations of MAbs directed against epitope located around the cystine knot (β1) and against those encompassing the top of loops 1 and 3 on hCGβ (β2, β4). Most epitopes recognize antigenic determinants on the protein structure of hCG. Only two well-characterized MAbs (B152 and CTP 104) detect carbohydrate molecular forms on hCG. The B152 antibody recognizes the biantennary core 2 O-glycan on

Ser132 and adjacent peptide structures, whereas CTP104 reacts with sialylated glycan on Ser138 [49, 64]. Availability of MAb B152, which was prepared using a purified urinary preparation of hCG (C5) that was 100% hyperglycosylated, enables direct measurement of HhCG.

1.17 Reference values in non-pregnant women

Appropriate reference values are critical for application of tests in patient management. Concentrations of hCG and its subunits in urine and serum samples of non-pregnant women and men without evidence of cancer have therefore been determined using ultra-sensitive time resolved immunofluorometric assays [120] (Table 6). The levels are about 3–10% of LH concentrations when expressed in U/L and exhibit a similar fluctuating pattern to LH. Furthermore, an increase in serum LH and FSH during the menopause is accompanied by a small increase in hCG levels. This suggests that most hCG in non-pregnant serum is derived from the pituitary. The genes for both hCG subunits are expressed in low levels in many tissues including thyroid, bladder, adrenals, breast and skeletal muscle, but it is not known whether hCG from these sources contribute to the serum hCG level [95].

The upper reference limit for hCG is 8.6 pmol/L (3 U/L) for women <50 years old and 15.5 pmol/L (5.3 U/L) in those ≥50 years old. [95] The upper reference limit for hCGβ in women <50 years is 1.6 pmol and in women ≥50 years old is 2.0 pmol/L. hCGβcf levels in serum are below the detection limit of current assays. In urine, hCG and hCGβ concentrations are on average 50% of those in plasma but there is considerable hCG immunoreactivity due to hCGβcf, as urine levels are ~4000-fold higher than serum levels. Upper reference limits of hCGβcf for pre- and post-menopausal women are 8.1 and 9.5 pmol/L, respectively. The α-subunit is mainly derived from pituitary, and its serum concentration does not reflect hCG production [95].

	<50 years		≥50 years	
	Pmol/L	U/L	Pmol/L	U/L
Serum				
HCG	8.6	3.0	15.5	5.4
HCGβ	1.6		2.0	
Total HCG	9.0		17.0	
Urine				
HCG	8.8	3.1	11.5	4.0
HCGβ	1.7		4.3	
HCGβcf	8.1		9.5	
Total HCG	13.6		20.4	

Table 6 Upper reference limits for serum and urine concentrations of hCG, hCGß and hCGßcf in non-pregnant women [95]

1.18 Study aims

The primary aims of the work presented in this thesis are

- To compare the levels of hCG and HhCG in uneventful pregnancies with those observed in pregnancies with complications.
- To investigate the diagnostic accuracy of HhCG in the prediction of adverse pregnancy outcome, comparison this with that of other hCG molecular forms including hCG, hCGβ and hCGβcf.
- To study the sugar chain heterogeneity of purified hCG from different sources using lectins and MAbs and to gain an insight into the spectrum of hCG glycosylation patterns during different stages of pregnancy and in pregnancy complications.

CHAPTER 2. MATERIAL AND METHODS

2.1 Clinical studies

2.1.1 Study patients

Patient cohorts are described in detail in the relevant chapters. A University of Edinburgh statistician was consulted about study design and statistical requirements at an early stage. She advised that as these are observational cohort studies, intended to establish levels of the relevant analytes across the clinical spectrum, minimum numbers of patients per cohort cannot be defined.

2.1.2 Ethical approval

Patient studies were approved by the local Ethics Committee. In all cases informed consent was obtained in accord with the local requirements. [See Appendix for copies of Patient Information, Patient Consent and Ethics forms.]

2.1.3 Specimen collection

Blood samples were collected into plain collection tubes and sent to the laboratory within two hours of collection. Serum was separated by centrifugation (2000 g) and aliquoted into appropriately labelled sterile microtubes (2 x 1 mL). They were stored at -20°C temporarily prior to being transferred for long-term storage to at -80°C.

Urine samples were collected in wide mouthed sterile containers and sent together with serum samples from the same patient to the laboratory. Following addition of preservative (0.05% w/v sodium azide), urine specimens was aliquoted into microtubes (6 x 1 mL) and stored as for the serum samples.

2.1.4 Specimen transport

Stored samples were transferred on dry ice from the UAE to the Department of Clinical Biochemistry, Royal Infirmary of Edinburgh. There they were kept at -20°C and thawed in batches at room temperature immediately prior to analysis. Similar transport arrangements were used to send serum and urine samples to Quest Diagnostics, Nichols Institute, USA. In both cases samples were kept in dry ice during transit by the courier and remained frozen until immediately before they were assayed.

2.1.5 Expression of results for hCG molecular forms in molar units

In order to facilitate comparability of results for different hCG molecular forms, results are generally expressed in pmol/L. Concentrations in terms of IU of the 4th IS 75/589 has been converted to a molar equivalents based on the biological activity of the standard (Table 1).[197]. Thus hCG assay results, obtained in U/L, were converted to pmol/L by multiplying them with 2.9. HhCG mass values (i.e. ng/ml or 1 μg/L) were converted to molar equivalent of hCG using the following calculation: 1 μg/L of HhCG = 24.39 pmol/L, based on the MW of 41000 Daltons [213].

2.1.6 Corrections for specificity gravity (urine samples)

As spot urine samples exhibit a wide range of densities due to hydration status and the time since last micturition [214], the hormone levels in urine samples were normalized using the method of Alfthan et al. [215] (reviewed in [214]), correcting hCG concentrations using the equation hCG corrected = hCG raw × (SpG sample -1) /(SpG target -1), where SpG sample is the specific gravity of the sample, and SpG target is a target specific gravity. We used 1.015 as the target specific gravity after calculating specific gravidity means from our study population, reflecting generally the low density of UAE-based population urine samples compared to those of Western populations [214].

2.2 Time-resolved immunofluorescence immunoassays

Serum and urinary HhCG, hCGβ, and hCGβcf of hCG (hCGβcf) were all quantified by ultra-sensitive time-resolved immunofluorometric assay using in-house assay reagents (kindly provided by Professor U-H Stenman and Dr H Alfthan, Helsinki University Central Hospital) on the DELFIA automated assay platform (Perkin-Elmer, Turku, Finland). The same system was used for measurement of hCG, using commercially available reagents. The experimental protocols are described below and summarized in Table 7.

2.2.1 Immunofluorometric immunoassay for HhCG

Capture antibody. Solid-phase antibody was prepared by physical adsorption of purified MAb B152 (4 μg/mL) in buffer (0.1 mol/L sodium carbonate; pH 9.3) onto the walls of polystyrene microtitre wells.

Detection antibody. Europium-labelled antibody (F95-1B2) was prepared and kindly gifted by Dr Alfthan. This antibody recognizes the hCGβ region in hCG.

Immunoassay procedure. Assay buffer (150 µL of 0.1M, pH 7.8 Tris-HCl buffered saline containing 5 g/L bovine serum albumin, 0.5 g/L bovine globulin, 0.15 mol/L sodium azide and 0.1 g/L Tween-40) was pipetted into each of 96 microtitre wells coated with MAb B152 as described above. Duplicate aliquots (50 µL) of standard (0, 9, 90, 900 and 9000 pmol/L of in-house standard prepared from a JEG3 choriocarcinoma cell line) or sample (serum or urine) was added to each well and the plates incubated for 2.5h at room temperature with continuous shaking ("Varishaker", Dynatech, UK).

Wells were washed four times with wash solution (assay buffer containing 0.5 mL/L Tween 20) using an automated twelve-channel washing device (Nunc-Immunowash 12, Roskilde, Denmark). Europium-labelled MAb 95/1B2 (50 ng in 200 µL of assay buffer) was added and the wells incubated for a further 30 minutes at room temperature. They were then washed six times with wash solution using an automated washing device. Enhancement solution (200 µL of 6.8 mmol/L potassium hydrogen phthalate, 0.1 mol/L acetic acid, 50 µmol/L tri-n-octylphosphine oxide, 15 µmol/L 2-naphtholyl trifluoroacetone and 1 g/L Triton X-100) was then added to the wells carefully, taking care not to touch the edge of the well so as to avoid contamination. The wells were then shaken for a few minutes and fluorescence was then measuring using an Arcus 1230 fluorimeter (Wallac Biochemical Laboratories).

Assay performance: The detection limit was 3 pmol/L of HhCG and the intra-assay CV 6%. Inter-assay CVs. were 9% at 47 pmol/L and 4% at 1100 pmol/L. Cross-reaction with hCG is 100%, with hCGβ 11% and with hCGβcf, hCGα, LH and FSH <0.1%. As there is no available IS for HhCG, a standard produced from conditioned medium from the JEG3 choriocarcinoma cell line was used as calibrant. These were in concentrations of 0, 9, 90, 900, 9000 pmol/L and was calibrated against DELFIA hCG kit standards (i.e. WHO 4th IRP 75/589).

2.2.2 Immunofluorometric immunoassay for hCGβ

Capture antibody. Solid-phase antibody was prepared as described in Section 2.2.1 using MAb F19-9C11 (4 µg/mL), which had been purified as previously described from mouse ascites [120].

Detection antibody. Europium-labelled antibody (1B2), which is specific for the hCGβ region of hCG, was prepared and gifted by Dr H Alfthan.

Immunoassay procedure. The procedure was exactly as described in Section 2.2.1. Assays standards contained 0, 0.73, 7.3, 73, 730 and 7300 pmol/L of added purified hCGβ and had been calibrated against hCG IS 75/589.

Assay performance: The detection limit of the assay was 0.2 pmol/L of hCGβ. The intra-assay CV was 12%. Inter-assay CVs. were 7% at 1.9 pmol/L and 3% at 27 pmol/L. Cross-reaction with HhCG is 6%, and with intact hCG, hCGβcf, hCGα, LH and FSH <0.1%.

2.2.3 Immunofluorometric immunoassay for hCGβcf

Capture antibody. Solid-phase antibody was prepared as described in Section 2.2.1 using MAb F52-3C11) (4 μg/mL), which had been purified as previously described from mouse ascites [120].

Detection antibody. Europium-labelled antibody (F95-1B2), which is specific for the hCGβ region of hCG, was prepared and gifted by Dr H Alfthan.

Immunoassay procedure. The procedure was exactly as described in Section 2.2.1 but only urine samples were assayed. The assay standards contained 0, 2, 20, 100, 200 500 and 2000 pmol/L of added purified hCGβcf and had been calibrated against hCG IS 75/589.

Assay performance: The detection limit of the assay was 0.2 pmol/L of hCGβcf. The intra-assay CV was 7%. Inter-assay CVs. were 10% at 7 pmol/L and 4% at 20 pmol/L. Cross-reaction with hCG, HhCG, hCGβ, hCGα, LH and FSH are all <0.3%.

2.2.4 Immunofluorometric immunoassay for hCG

Capture antibody. Wells coated with solid-phase antibody (MAb 5008) were purchased from Perkin-Elmer (Turku, Finland).

Detection antibody. Europium-labelled antibody (MAb 5501) was purchased from Perkin-Elmer (Turku, Finland). This antibody recognizes the hCGα region in hCG.

Immunoassay procedure. Assay buffer (200 μL of 0.1M, pH 7.8 Tris-HCl buffered saline containing 5 g/L bovine serum albumin, 0.5 g/L bovine globulin, 0.15 mol/L sodium azide and 0.1 g/L Tween-40) was pipetted into each of 96 microtitre wells. Duplicate aliquots (25 μL) of standard (0, 2, 10, 100, 1000, 5000 and 10,000 U/L of IS 75/589) or sample (serum or urine) was added to each well and the plates incubated for 2.5h at room temperature with continuous shaking ("Varishaker", Dynatech, UK).

Wells were washed four times with wash solution (assay buffer containing 0.5 mL/L Tween 20) using an automated twelve-channel washing device (Nunc-Immunowash 12, Roskilde, Denmark). Europium-labelled MAb 95/1B2 (50 ng in 200 µL of assay buffer) was added and the wells incubated for a further 30 minutes at room temperature. They were then washed six times with wash solution using an automated washing device. Enhancement solution (200 µL of 6.8 mmol/L potassium hydrogen phthalate, 0.1 mol/L acetic acid, 50 µmol/L tri-n-octylphosphine oxide, 15 µmol/L 2-naphtholyl trifluoroacetone and 1 g/L Triton X-100) was then added to the wells carefully, taking care not to touch the edge of the well so as to avoid contamination. The wells were then shaken for a few minutes and fluorescence was then measuring using an Arcus 1230 fluorimeter (Wallac Biochemical Laboratories).

Assay performance: The detection limit of the assay was 0.2 U/L of hCG, and the intra-assay CV <10%. Inter-assay CVs. were 3%, 2% and 2% at 6.1 U/L, 19 U/L and 284 U/L respectively pmol/L. Cross-reactions with HhCG is 6%, <0.1% for hCGβ, hCGβcf and hCGα, and <0.3% for LH and FSH.

Methods	HhCG	hCG	hCGβ	hCGβcf (Urine only)
Standards	4 standards (JEG3 cell line)	7 standards	5 standards	5 standards
Calibration	hCG IS 75/589	hCG IS 75/589	hCG IS 75/589	hCG IS 75/589
Volume of standards and samples	50 µL	25 µL	50 µL	50 µL
Volume of buffer	150 µL	200 µL	150 µL	150 µL
Incubation time	2½ hours	1 hour	2 hours	2 hours
Number of washes	x 2	x 2	x 2	x 2
Volume of tracer	200 µL	200 µL	200 µL	200 µL
Incubation time	30 min.	30 min.	30 min.	30 min.
Number of washes	x 4	x 4	x 4	x 4
Volume of enhancement solution	200 µL	200 µL	200 µL	200 µL
Time on slow shaker	5 min.	5 min.	5 min.	5 min.
Check concentration by measuring fluorescence				

Table 7 Summary of sequential steps in the immunofluorometric assays used

2.3 Chemiluminescent immunoassays

HhCG and hCG were measured in serum and urine using commercially available methods with chemiluminescent labels.

2.3.1 Chemiluminescent immunoassay for HhCG

HhCG was measured in serum and urine using the Quest Diagnostics immunoassay (San Juan Capistrano, California). The assay uses MAb B152 as capture MAb and an anti-hCGβ mouse MAb labelled with chemiluminescent acridinium ester as a detection antibody. The protocol is as previously described [76].

Assay performance: The detection limit of the assay was 0.2 µg/L of HhCG. The intra-assay CV was 7%. Inter-assay CVs. were 10% at 7 pmol/L and 4% at 20 pmol/L. Cross-reactions with HhCG are HhCGβ are 100% and 60% respectively and <5% with hCG, hCGβn and their nicked forms. The assay is calibrated in mass units against an HhCG standard purified from a JEG3 choriocarcinoma cell culture.

2.3.2 Chemiluminescent immunoassay for hCG

HCG was measured in serum and urine using the Siemens Immulite hCG assay (Siemens Diagnostics, Los Angeles). The assay is designed for the quantitative measurement of total hCG (i.e. hCG, hCGβ and their nicked forms. Details of the method are readily available.

The detection limit of the assay is 1 U/L of hCG. The intra-assay CV ranged from 3.6 to 5.2% for concentrations between 3330 and 30 U/L respectively, while inter-assay CVs. ranged from 7.8 to 9.9% for concentrations between 37 and 3569 U/L respectively.

2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used technique for the separation of proteins according to molecular weight. It can be applied to molecules with molecular weight ranging from 10-250 kDa. SDS-PAGE was performed as described below.

2.4.1 Preparation of samples for SDS-PAGE

Protein samples were prepared in both non-reduced and reduced forms. For non-reduced samples, protein sample (approximately 0.5 mg/L; 70 μL) and sample buffer [Novex NuPAGE™ LDS Sample Buffer (Invitrogen Life Sciences, Carlsbad, California), diluted four times with distilled water; 50 μL] were mixed with distilled water (100 μL). Reduced protein samples (approximately 0.5 mg/L; 70 μL) were prepared by adding β-mercaptoethanol (5% v/v in sample buffer; 7 μL). Non-reduced and reduced sample mixtures were then boiled at 70°C for 10 minutes.

2.4.2 Electrophoresis procedure

Reduced and non-reduced samples (purified hCG from Pregnyl or JEG3 cell lines) were loaded into separate wells in pre-cast polyacrylamide gradient gels (NuPAGE™ Novex Bis-Tris mini-gels, 4-12% 2D well and 1% 12D well). Molecular weight markers (Bio-Rad; 5 μL) were loaded in the same way. Electrophoresis was then performed for 30-40 minutes at a constant voltage of 200 V in a vertical slab gel apparatus (Xcell™ Surelock electrophoresis cell; Invitrogen Life Sciences, USA). The

inner and outer chambers of the cells were filled with running buffer [NuPAGE™ MES SDS Running Buffer (Invitrogen Life Sciences, Carlsbad, California) diluted twenty times with distilled water; 50 mL] until the molecular weight markers reached the bottom of the gel. Gel casts were then opened carefully to release the gels and immediately soaked in running buffer.

2.5 Western blotting

Western blotting involves electrotransfer of proteins to a solid supporting membrane, usually nitrocellulose, which can then be probed with either antibodies or lectins to determine different immunoreactivities or glycosylation patterns. The procedures used are described below.

2.5.1 Protocol for Western blotting

Four sheets of absorbent filter paper and one sheet of nitrocellulose membrane (Pore size 0.45; Hybond ECL™, Amersham Biosciences, Germany) were cut to the size of the gel. The nitrocellulose membranes and support pads were saturated with transfer buffer [NuPAGE Transfer Buffer (Invitrogen Life Sciences, Carlsbad, California) diluted twenty times in water containing 20% v/v ethanol]. A transfer sandwich was assembled in blot module by layering two support pads, one sheet of filter paper, one nitrocellulose membrane, one polyacrylamide gel, one sheet of filter paper and two support pads in that order. For transferring two gels in one blot module, the second sandwich was separated from the first by adding one or two more support pads. All the components were kept wet, making sure that good contact was established between the gel and the membrane with no trapped air bubbles. The complete transfer sandwich was placed in the transfer tank with the membrane closest to the anode. Electroblotting was performed at 40v for one hour [XCell II™ Blot Module (Invitrogen Life Sciences, Carlsbad, California)].

2.5.2 Antibody probing of Western blots

After electrophoresis transfer was completed, the nitrocellulose membranes were cut into appropriately sized strips and labelled with a ballpoint pen. The strips were then gently rocked in troughs containing blocking buffer [5% w/v non-fat dried milk powder (Marvel) in phosphate buffered saline containing 0.1% Tween-20] for 1h at room temperature in order to block unoccupied binding sites and prevent non-specific

adsorption of antibody. The strips were then washed with PBS containing Tween-20 (1% v/v) three times at 5 minute intervals. The strips were then incubated for 1h with the first antibody (1:5000 in blocking buffer) which binds to proteins immobilized on the membranes. The MAbs used were B152 (gift from Dr S Birken, Columbia University, NY; specific for HhCG) and hCG.8F11 SMA (Mologic, Bedford, UK; specific for hCGβ).

The membranes washed as described above and were then incubated for a further 1h with goat (for MAb B152) or donkey (for MAb hCG.8F11SMA) anti-immunoglobulin antibody coupled to alkaline phosphatase (1:5000 in blocking buffer). They were then washed again in the same way.

Following addition of enzyme substrate [200 μL of nitroblue tetrazolium chloride (NBT, Sigma, UK; 50 mg/mL in dimethylformamide) and 100 μL of 5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt (BCIP, Sigma, UK; 50 mg/mL in dimethylformamide) in 30 mLs of Tris-HCl buffer (0.05M, pH 9.5)], deep purple bands appear within 10-20 minutes. The reaction was stopped by immersing the strips in distilled water, after which the membrane was allowed to dry prior to scanning (Hewlett-Packard Printer Digital Scanner 2200).

2.5.3 Lectin probing of Western blots

Following electrophoresis, lectin probing was performed essentially as described for MAbs in Section 2.5.2, but using different wash (0.5% w/v BSA in PBS containing 0.2% v/v Tween-20) and blocking (2% w/v bovine serum albumin in PBS) buffers. Lectins (0.1% w/v; 1:1000 in blocking buffer) were used instead of MAbs. The second incubation used streptavidin alkaline phosphatase conjugate (Sigma, UK; 1:5000 in blocking buffer) following by reaction NBT/BCIP as described in Section 2.5.2. All lectins were obtained from Sigma, UK and are listed in Table 8.

2.5.4 Antibody and lectin probing of patient samples

Frozen urine samples were thawed at room temperature. Creatinine was measured in the Department of Clinical Biochemistry, Royal Infirmary of Edinburgh, using a commercially available enzymatic method (Alpha Laboratories Ltd, Eastleigh UK) adapted from that of [216] for use on the Cobas Fara centrifugal analyzer (Roche Diagnostics Ltd, Welwyn Garden City, UK) to ensure concentrations were within

normal reference limits. Within- and between-run CVs. were <3% and <5% respectively.

Using hCG results obtained with the Perkin-Elmer DELFIA method [Section 2.2.4], concentrations of hCG in the urine samples were diluted to concentrations between 9,690 and 19,317 U/L.

SDS-PAGE and Western blotting were performed as described in Sections 2.4 and 2.5.1 and probing with antibodies and lectins were performed as above (Sections 2.5.2 and 2.5.3).

No.	Code	Lectin	Abbreviation
1	2	*Lens culinaris* agglutinin	LCA
2	3	Wheat germ agglutinin	WGA
3	4	*Galanthus nivalis* agglutinin	GNA
4	6	Jacalin	J
5	7	Soya bean agglutinin	SBA
6	9	*Sambucus nigra* agglutinin	SNA
7	10	*Lotus tetragonolobulus* lectin	LT
8	11	*Amaranthus caudatus* lectin	ACL
9	12	*Solanum tuberosum* lectin	ST
10	14	*Datura stramonium* agglutinin	DSA
11	15	Peanut agglutinin	PA
12	18	Concanavalin A lectin	Con A
13	19	*Dolichos biflorus* agglutinin	DFA
14	20	*Ulex europaeus* agglutinin I	UEA
15	21	*Phaseolus vulgaris* leucoagglutinin	PHA-L
16	22	*Pisum sativum* agglutinin	PSA
17	23	*Saphora japonica* agglutinin	SJA
18	24	*Griffonia simplicifolia* I lectin	GS-I
19	25	*Phaseolus vulgaris* erythroagglutinin	PHA-E
21	27	Succinylated wheat germ agglutinin	WGA-S
22	29	*Vicia villosa* lectin	VV
23	31	*Lycopersicon esculentum* lectin	LE
24	32	*Erythrina cristagalli* lectin	EC
25	33	*Griffonia simplicifolia* II lectin	GS-II
26	34	*Lathyrus odoratus* lectin	LO
27	35	Alkaline phosphatase: streptavidin	Control

Table 8 List of lectins used for protein probing

2.6 Statistical analyses

2.6.1 Data analysis and statistical packages

Statistical analysis was performed using MINITAB (Versions 13 and 14) and S-plus statistical packages. Normally distributed data are described as means ± standard deviation (SD) or means and ranges. Non-normally distributed data are described as medians with the interquartile ranges (i.e. 25^{th} - 75^{th} percentiles) (Q1, Q3). Serum and

urinary concentrations of all hormonal analytes showed a wide range of distribution; hence concentrations were log-transformed for easy comparison of data, and plotted against week of gestation. HCG ratio measurements are displayed on a linear scale.

Comparisons between the groups were made using either two-tailed t-test or non-parametric Mann-Whitney or Kruskal-Wallis tests as appropriate. P values below 0.05 were considered to be significant. Boxes in the box plots define the 25^{th} to 75^{th} percentiles. The upper and lower bars indicate the 90^{th} and 10^{th} percentiles, respectively. A line inside the box marks the 50^{th} percentile value. Lines extending from upper and lower bar (i.e. whiskers), represent the values, 1.5 times above and below the range of hormonal data. All asterisks indicate values either lower or higher than the whisker values.

2.6.2 Receiver-operating characteristics (ROC) curves

Receiver-operating characteristics (ROC) curves and the area under the curves (AUC) were calculated to assess the diagnostic accuracy of each test for these analytes. ROC curves represent the full spectrum of possible sensitivity-specificity pairs to evaluate a test for possible clinical application, with the y-axis representing sensitivity (true positive) and the x-axis representing 1-specificity (true negative).

The model created by Duc et al. [217] was used to rank the accuracy of a diagnostic test. They rated AUC values of 0.91-1.00 and 0.0-0.09 as excellent accuracy (++), 0.81-0.90 and 0.10-0.19 as good accuracy (+), 0.61-0.80 and 0.20-0.39 as moderate accuracy (±), and 0.40-0.60 as poor accuracy (-) [217].

The performance of various hormonal cut-off values was tested to maximize sensitivity and specificity and expressed using the following definitions [218]:

Sensitivity: The proportion of patients identified as a viable pregnancy (singletons and twins) when the HhCG cut-off value is above the cut-off point (true negatives).

Specificity: The proportion of women identified as a nonviable pregnancy when an HhCG value is below the cut-off point also referred to as true positives.

Positive predictive value: The probability that a patient with an HhCG value less than the cut-off will have an early pregnancy loss.

False-positive rate: The percentage of women with an HhCG value less than the cut-off, who have a viable pregnancy.

False-negative rate: Percentage of miscarriage cases that will be missed by the chosen cut-off point (i.e. nonviable pregnancies wrongly identified as a viable one).

CHAPTER 3. HYPERGLYCOSYLATED HCG AND OTHER HCG MOLECULAR FORMS IN SPONTANEOUS PREGNANCY AND PREGNANCY RELATED DISORDERS (THE UAE POPULATION)

3.1 Background to the Clinical Study in the UAE

Since the development of MAbs against various molecular forms of hCG, the association between abnormal levels of hCG and adverse pregnancy outcome can be studied using a range of sensitive biochemical immunoassays. In addition to triploidy and multiple gestations, the elevation of hCG level has been associated with a series of obstetric complications that include pre-eclampsia, small for gestational age (SGA), preterm delivery, and neonatal death. Reports of diminished hCG levels have also been linked with unfavourable pregnancy outcome [113, 125, 219-225]. Despite such associations, the clinical use of hCG analytes as a biochemical screening marker of adverse pregnancy events is limited. Current obstetric practice uses second-trimester maternal serum levels of hCG along with AFP, and unconjugated oestriol as a screening tool for fetal aneuploidy [226-232]. hCG quantitation is also used in diagnosing spontaneous abortion, ectopic and multiple pregnancy, detecting and following up hCG-producing tumors, including trophoblastic disease and certain testicular and ovarian tumors [207, 212, 226, 233-243].

HhCG, with additional sugar residues on its oligosaccharide side chains, is produced predominantly by phenotypically invasive cytotrophoblast cells. Higher levels of HhCG are associated with periods of invasive events during the phase of implantation, and with gestational trophoblastic disease [69, 70, 130, 244]. Trophoblastic abnormalities and defective implantation have been previously implicated in the origin of several pregnancy disorders, including spontaneous abortion, pre-term delivery, pregnancy-induced hypertension (PIH) and intra-uterine growth retardation. It therefore seems plausible that abnormal levels of HhCG in maternal serum or urine may provide an early indication of placental dysfunction and early identification of failing pregnancy or any other pregnancy complication. This is important in order to provide appropriate clinical care and counselling before the onset of disease or pregnancy failure. So far, no parameters have been described that use measurements of HhCG to differentiate between favourable outcome and pregnancy failures. The following study aims at evaluating 1st and 2nd trimester HhCG levels in normal and pathological pregnancies in order to establish its clinical application for the detection of adverse pregnancy outcome. Also, the levels of HhCG were compared with the

levels of hCG, hCGβ, and hCG βcf, in order to determine whether it is a better indicator of pregnancy outcome than other hCG-related molecular forms.

3.2 Study protocol

Pilot prospective cohort study

Pregnant women attending the Antenatal Clinic, In-patient Obstetrics and Gynaecology wards, and Accident and Emergency Department at Mafraq Hospital, which is a 500 bed multi-referral centre in Abu Dhabi, United Arab Emirates.

3.2.1 Ethics

Full ethical approval had been obtained from the Ethics Committee at the Mafraq Hospital before commencing the study. A signed consent was obtained before enrolling the patient (refer to appendix).

3.2.2 Study group

292 pregnant women (age range 15-49 years) belonging to a multi-ethnic community including Arabs, Asians and expatriate Westerners were included in this study. Only spontaneous singleton pregnancies were considered for this study. The gestational age was calculated from the first day of last menstrual period, unless ultrasonography before 16 weeks demonstrated a discrepancy of at least 10 days, in which case U/S dating was used for the calculations. The patients were followed until the outcome of their pregnancies was known.

3.2.3 Data collection

Between June 2003 and June 2004, 164 first trimester (6-12 weeks) and 136 second trimester (13-24 weeks) urine and serum samples were collected for this study. The outcome data were later retrieved from patient records.

Measurement of hCG and related molecular forms was performed in serum and urine as described in Chapter 2. Unless otherwise stated, all hormonal values are expressed in pmol/L (medians with the interquartile ranges). Statistical significance was defined as $p<0.05$.

3.3 Denominators of outcome variables [245]

3.3.1 Uneventful pregnancy

Spontaneous singleton pregnancies progressed to term (≥37 weeks) without any complications and resulted in the live birth of an infant weighing ≥2500 gms with no signs of chromosomal or structural abnormality.

3.3.2 Spontaneous miscarriage

According to the criteria of the World Health Organization (WHO, 1977), spontaneous abortion is defined as the expulsion of an embryo (blighted ova) or extraction of a fetus weighing 500g or less. Although this fetal weight will normally correspond to a gestational age of 20-22 weeks, in Mafraq Hospital due to the lower survival rate, pregnancy losses up to 24 weeks was included in spontaneous miscarriage group.

For descriptive purposes, miscarriages were classified according their subtypes depending on the clinical picture at the time of sample collection, but all the subtypes eventually ended in spontaneous miscarriage, either spontaneously or after following medical and/or surgical intervention. This group includes symptomatic patients presenting with vaginal bleeding (with or without abdominal pain) and a live fetus with the cervix undilated but who subsequently progressed to spontaneous miscarriage. The clinical and ultrasonographic diagnosis at the time of serum and urine sample collection was threatened miscarriage. The non-threatened miscarriage group included women who at the time of sample collection had a live viable pregnancy with no bleeding, with or without abdominal pain (asymptomatic), but who eventually had a spontaneous miscarriage. The missed miscarriage group included women whose pregnancy failure was identified before the expulsion of products of conception which were either embryonic (i.e. gestation sac and fetal pole seen but no fetal heart, in ultrasound) or anembryonic (i.e. gestation sac seen with no fetal pole on U/S). Women with incomplete miscarriage were those who passed the products of conception incompletely, with evidence of retained tissue in the uterus. Finally, the inevitable miscarriage group included women who had bleeding prior to 24 weeks associated with pain and dilatation of cervix.

3.3.3 Ectopic pregnancy

Ectopic pregnancy occurs when a fertilized ovum is implanted outside the uterine cavity. All these patients, at the time of blood and urine test, had an unruptured tubal pregnancy confirmed on ultrasound and/ on laparoscopy.

3.3.4 Molar pregnancy (hydatidiform mole)

Oedematous and avascular villi and trophoblastic overgrowth was present. The classical "complete" mole had no fetus. "Partial" moles had focal molar changes in the placenta. A fetus was sometimes present.

3.3.5 Proteinuric and non-proteinuric pregnancy-induced hypertension

PIH was defined as diastolic blood pressure of ≥90 mmHg or a systolic presuure of ≥140 mm Hg on at least two consecutive occasions, 6 hours apart, in a woman with no history of pre-existing hypertension or renal disease, and after the 20th weeks of gestation. Patients with PIH were further subdivided into non-proteinuric and proteinuric ((i.e. gestational hypertension in the setting of significant proteinuria, defined as a minimum of 300 mg/24 hours or 0.1 g/L in at least 2 random samples that were collected ≥6 hours apart) and included pre-eclampsia/and or eclampsia. Pre-eclampsia is the development of hypertension with proteinuria or edema (pitting edema). Eclampsia is the occurance of convulsions in a woman with symptoms of severe pre-eclampsia such as visual disturbances, epigastric pain, oliguria, etc [246].

3.3.6 Small for gestational age

Small for gestational age (SGA) was defined as a birth weight 10th or less percentile for gestational age.

3.3.7 Preterm delivery

Pre-term delivery (PTD) was defined as a delivery occurring before 37 completed weeks of gestation which included spontaneous preterm labour and or premature rupture of membrane, or a delivery induced, medically or surgically, due to a maternal or fetal cause.

3.3.8 Antepartum haemorrhage

Antepartum haemorrhage (APH) was defined as bleeding from the genital tract between the 28th week of pregnancy and the onset of labour, and included placenta

previa (defined as a placenta in which the lowermost edge covered the internal os of the cervix, either in whole or in part at the time of delivery) and placental abruption (defined as premature separation of normally sited placenta).

3.3.9 Gestational diabetes mellitus

The diagnosis of gestational diabetes mellitus (GDM) is based on a positive glucose challenge test, which according to current departmental protocols in Mafraq Hospital, is performed for all pregnant women between 18th to 24th pregnancy weeks. The two-stage screening guidelines of the American College of Obstetricians and Gynaecologists (ACOG) (i.e. a non-fasting post 50-g, 1-h plasma glucose screen followed by the confirmatory 'gold standard' 100-g, 3-h oral glucose tolerance test as a positive screen [247].

3.3.10 Amniotic fluid index abnormality

Abnormalities in the amniotic fluid volume were determined by using ultrasound to measure the sum of the deepest pockets of amniotic fluid in the 4 quadrants of the maternal abdomen to derive the amniotic fluid index (AFI). These abnormalities include oligohydramnios (an amniotic fluid index $\leq$ 5 cm), polyhydramnios (AFI greater than 25 cm), and anhydramnios (absence of amniotic fluid)

3.3.11 Macrosomia

Macrosomia was defined as a birth weight of >4000 grams.

3.3.12 Intrauterine fetal death or neonatal death

As per the Obstetrics and Gynaecology Departmental protocol in Mafraq Hospital, the official definition of intrauterine fetal death or still birth is a child that has issued forth from its mother after the 24th week of pregnancy and which did not at any time after being completely expelled from its mother breathe or show any other signs of life (given in Section 41 of the Births and Deaths Registration Act 1953 as amended by the Stillbirth Definition Act 1992) and/ or deaths occurring *in utero* in which the fetus or neonate weighs 500g or more. Early neonatal death is demise of a live born baby occurring less than 7 completed days from the time of birth. The two are considered together in this study.

3.3.13 Miscellaneous conditions

These included pregnancies coexisting with medical conditions such as diabetes mellitus, hypertension and thyroid disorders.

3.4 Results for the UAE cohort

3.4.1 Patient outcome variables

A total of 289 patients were included in this study. 283 (98%) patients had matched serum-urine samples taken between 4 and 24 weeks of gestation. Only 6 patients (2%) did not have paired serum or urine samples collected. [An additional 4 cases were excluded due to non-availability of the outcome data.]

The 289 patients were classified into two groups:

Group 1 ("Continuing group"): 217 patients (75%) whose pregnancy continued beyond 24 weeks.

Group 2 ("Non-continuing group"): 72 patients (25%) whose pregnancies ended before 24 completed weeks of gestation due to spontaneous miscarriage, ectopic pregnancy or molar pregnancy.

Table 9 compares the demographic variables between the two groups. Only in respecdt of age and parity were there statistically significant differences, with other parameters comparable in both groups.

Patient characteristics	Group 1 N= 217	Group 2 N= 72	P Value
Age			
• ≤35	188	50	<0.05
• >35	27	21	
BMI			
• <25	89	27	NS
• ≥25	126	44	
Parity			
• Primi	81	20	
• Multi (1-5)	100	29	<0.05
• Grand-multi (≥5)	36	23	
Ethnicity			
• UAE	93	36	
• GULF	67	18	
• N.AFRICA	23	11	NS
• SE. ASIA	32	7	
• OTHER	2	0	

Table 9 Demographic characteristics of the study group. [NS, Difference not statistically significant]

The "Continuing group" included 137 uneventful pregnancies (i.e. term deliveries with no complication), constituting 63% of this group and 47.4% of the total study population. The remaining 37% had adverse pregnancy complications, occurring either as an isolated condition or in combination with other complications. Table 10 shows outcome variables of the "continuing group" and their incidences in study population.

Outcome variables	N	% of group 1 N=217	% of total study group N=289
Uneventful pregnancy	137	63.1	47.4
Threatened bleeding	18	8.2	6.2
Gestational Diabetes	14	6.4	4.8
Non-proteinuric + proteinuric PIH	14	6.4	4.8
Fetal growth restriction	11	5	3.8
Preterm delivery	18	8.2	6.2
• Spontaneous	8	3.6	2.7
• Induced	10	4.6	3.4
Ante-partum haemorrhage	3	1.3	1.0
AFI abnormality	5	2.3	1.7
Macrosomia	14	6.4	4.8
Intrauterine fetal death / neonatal death	8	3.7	2.7
Other	12	5.5	4.1

Table 10 Outcome variables in the "continuing group" and their incidences in the total study population as well as in relation to other outcome variable in the "continuing group". Note that the incidence of each complication is given irrespective of their co-existence with other pregnancy complications.

3.4.2 Hyperglycosylated hCG levels during uneventful pregnancy

Serum and urine HhCG concentrations for women with uneventful pregnancies in the "continuing group" (singleton pregnancy with no complication) are shown in Figures 24 and 25.

HhCG levels in the first and second trimester pregnancy samples for the 137 patients with uneventful pregnancy revealed higher levels of HhCG, in both serum and urine, during the 1st trimester when compared with the 2nd trimester (Table 11, Figures 24 and 25)

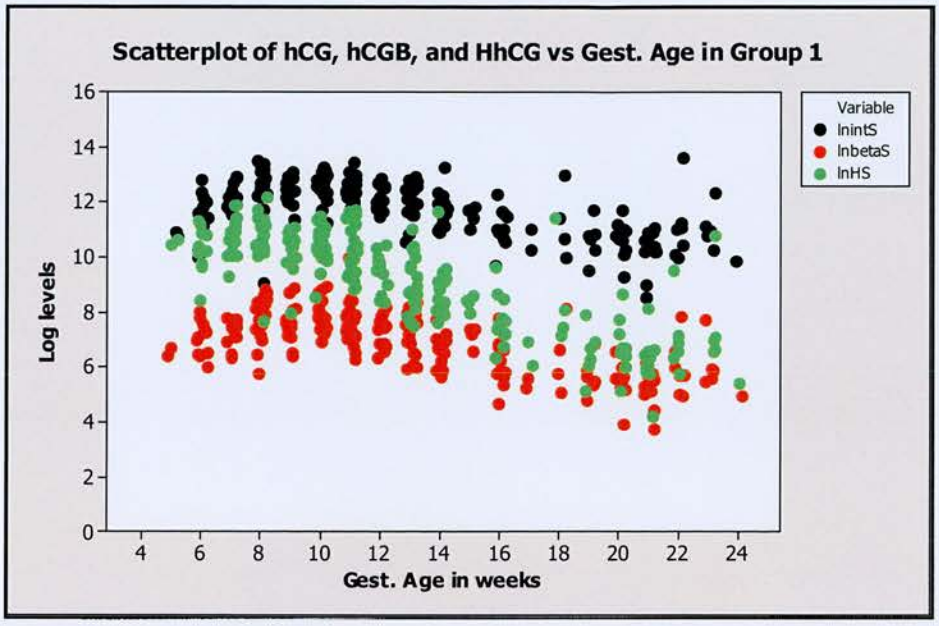

Figure 24 Display of log-transformed data of hCG (•), hCGβ (•), and HhCG (•) serum levels in from uncomplicated pregnancy in Group 1. lnintS, log *n* of intact hCG (serum); lnbetaS, log *n* of hCG beta-subunit (serum); lnHS, log *n* of hyperglycosylated hCG (serum).]

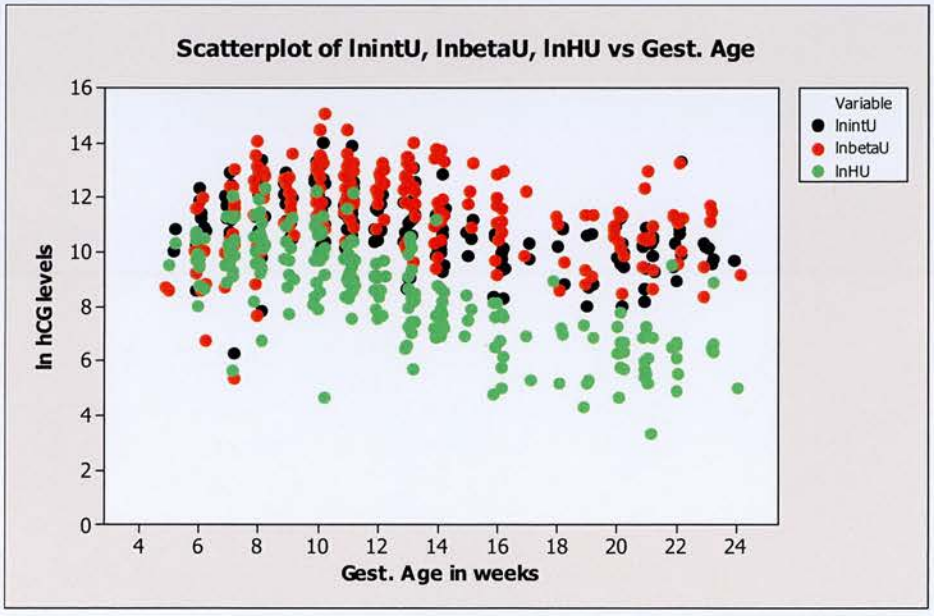

Figure 25 Display of log transformed data of hCG (•), hCGβ (•), and HhCG (•) urinary levels in from uncomplicated pregnancy in Group 1. [lnintU, log *n* of intact hCG (urine); lnbetaU, log *n* of hCG beta-subunit (urine); lnHU, log *n* of hyperglycosylated hCG (urine).]

		Gestational Age	N	Minimum	Q1	Median	Q3	Maximum
Serum	hCG	All	137	5118	70123	186850	301922	761372
		0-12	75	29491	180043	264818	383884	761372
		13-24	62	5118	43860	68257	159754	589206
	hCGβ	All	137	43.5	497	1347	2343	22430
		0-12	75	405	1267	2065	3006	22430
		13-24	62	43.5	256	417	1177	11985
	HhCG	All	137	66.1	3082	16144	39640	194592
		0-12	75	2890	18351	32572	66934	194592
		13-24	62	66.1	724	2114	7044	113853
Urine	hCG	All	135	2990	30725	67836	173664	1213527
		0-12	74	18681	67083	134377	257985	1213527
		13-24	61	2990	17511	32961	57648	490607
	hCGβcf	All	135	4463	43131	133354	362104	3490085
		0-12	74	6196	95972	223218	444784	3490085
		13-24	61	4463	29362	75677	213887	1253738
	HhCG	All	135	28.5	1143	7243	23544	228062
		0-12	74	110	9313	16709	35119	228062
		13-24	61	28.5	567	1078	3599	74378

Table 11 Serum and urinary concentrations of HhCG and other hCG-related forms in uneventful pregnancy (n=137). N= number of subjects, Q1 and Q3 represent quartile ranges of the value (i.e. 25^{th} -75^{th} percentiles)

The median (Q1, Q3) serum HhCG level during the 1^{st} trimester was 32,572 pmol/L (18351, 66934) and 2114 (724, 7044) pmol/L during the 2^{nd} trimester, indicating a 93.5% reduction in HhCG levels from the 1^{st} to 2^{nd} trimester. Changes observed in urinary HhCG concentrations were similar to those in serum. Median HhCG was 16709 (9313, 35119) pmol/L during the 1^{st} trimester and 1078 (567, 3599) during the second, with a 93.5% reduction in levels from the 1^{st} to 2^{nd} trimester of pregnancy. Overall, median urinary levels of HhCG were 45% lower than in serum. However, the variations in urine were found to be higher than in serum (28.5-228062 pmol/L and 66.1-194592 pmol/L, respectively).

The other four hCG analytes examined also exhibited a trend of high 1^{st} trimester concentrations followed by decreased 2^{nd} trimester levels, as for HhCG. HCG and hCGβ levels in serum during the 1^{st} trimester were 264818 (180043, 383884) pmol/L and 2065 (1267, 3006) pmol/L, respectively. During the 2^{nd} trimester, the median level for hCG and hCGβ decreased to 68257(43860, 159754) and 417(256, 1177) pmol/L, with a reduction of 74.3% and 80%, respectively. In urine, the levels of hCG and hCGβcf decreased from 134377 (67083, 257985) pmol/L and 223218 (95972,

444784) in the 1st trimester to 32961 (17511, 57648) pmol/L and 75677 (29362, 213887) pmol/L in the 2nd trimester, with a reduction of 75.5% and 66.1%, respectively (Table 11, Figures 24, 25).

The relationship between serum and urinary levels of HhCG and hCG were analyzed using Bland and Altman plots which show the difference of results (calculated as serum minus urine) for hCG and HhCG from serum samples plotted against hCG and HhCG in urine. For both plots, the mean of the difference between serum and urine results is greater than zero and most points lie above zero indicating, that serum results are consistently higher than urine results (Figure 26, 27).

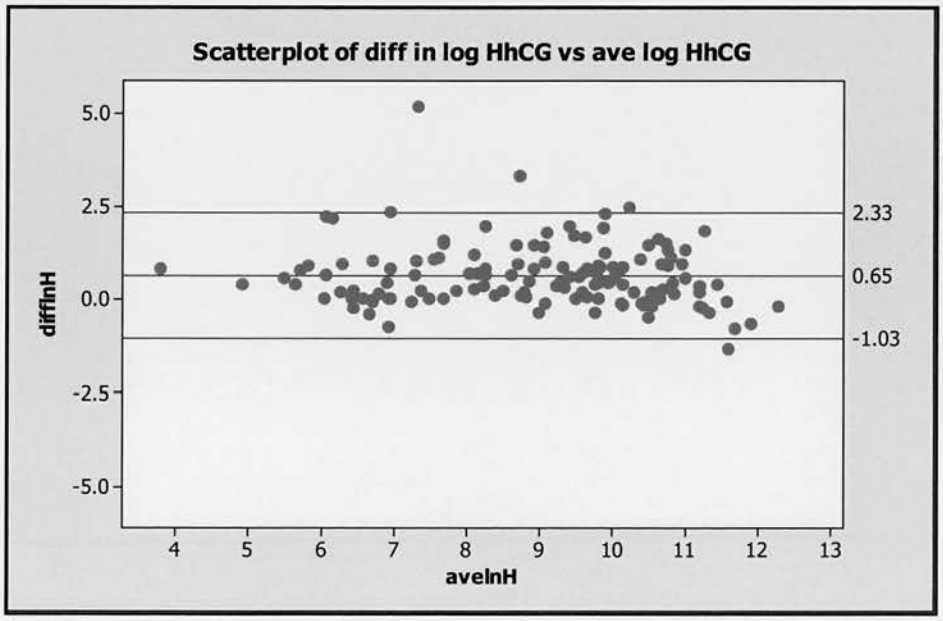

Figure 26 Bland and Altman plot showing higher levels of HhCG in serum than urine. The centre line indicates mean of difference and mean ± 2SD is represented by top and bottom lines.

Levels of HhCG were examined, in relation to other hCG molecular forms, in both serum and urine, by calculating their ratios (Table 12). In serum, the ratios of HhCG to hCG (HhCG/hCG) and HhCG to hCGβ (HhCG/hCGβ) were 0.11 and 16.5 during the 1st trimester and 0.03 and 4.4 during the 2nd trimester, respectively. In urine, the 1st trimester median molar ratios of HhCG to hCG and HhCG to hCGβcf were 0.14 and 0.09, and that of the 2nd trimester were 0.03 and 0.01, respectively. Although ratios decreased from the 1st to 2nd trimester, serum HhCG was lower than hCG but higher than hCGβ. In urine, hCG and hCGβcf levels were higher, as was total hCG in 1st trimester serum.

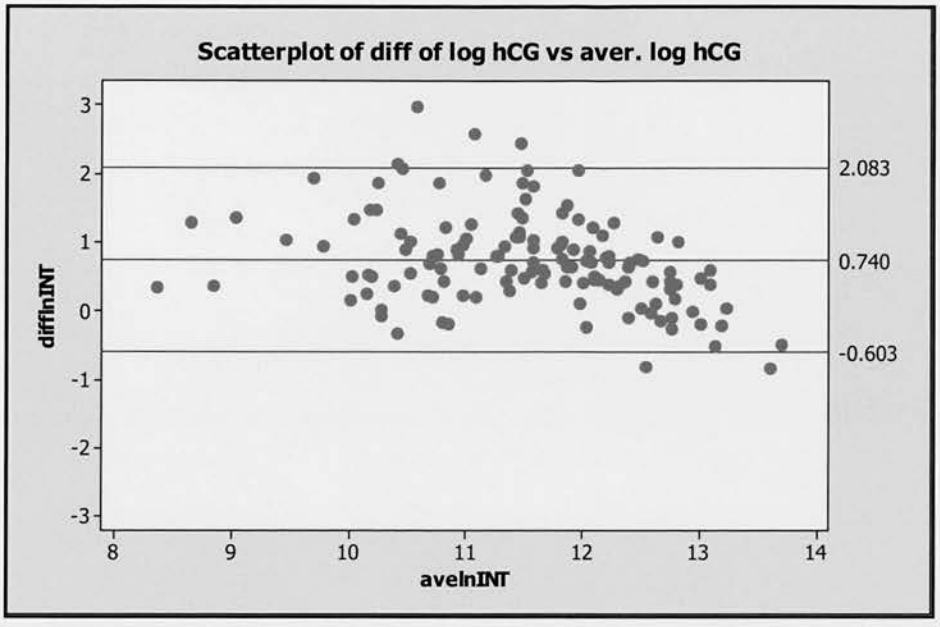

Figure 27 Bland and Altman plot showing higher levels of hCG in serum than urine. The centre line indicates mean of difference and mean ± 2SD is represented by top and bottom lines.

As total hCG immunoreactivity in serum included hCG+hCGβ, [69, 94, 120, 129, 248, 249], we examined the contribution of HhCG to total hCG immunoreactivity. HhCG comprised 11% of total hCG (Q1, Q3: 9%-21%, range: 3%- 60%) during the first trimester, whereas during the second trimester, the proportion decreased to 3% (Q1, Q3: 1%, 6%, range: 0.08%- 21%). Table 12 shows ratios of HhCG in relation to other hCG analytes in serum and urine of women with uncomplicated pregnancy during the 1st and 2nd trimesters.

		Gestational Age	N	Q1	Median	Q3
SERUM	HhCG/hCG	0-12	75	0.0907	0.1181	0.2208
		13-24	62	0.01779	0.03558	0.06332
	HhCG/hCGß	0-12	75	8.72	16.53	28.78
		13-24	62	3.057	4.462	8.903
	HhCG/(hCG+hCGß)	0-12	75	0.09	0.117	0.2195
		13-24	62	0.0177	0.03534	0.06294
URINE	HhCG/hCG	0-12	74	0.0916	0.1472	0.1962
		13-24	61	0.02537	0.03623	0.07466
	HhCG/hCGßcf	0-12	74	0.0463	0.0979	0.1876
		13-24	61	0.00844	0.017	0.0329

Table 12 Proportions HhCG in relation to to other hCG forms, expressed by calculating their ratios, in uneventful pregnancy (n=137).

3.4.3 HhCG levels in continuing vs. non-continuing pregnancy

The "non-continuing" group was comprised of 72 singleton pregnancies that ended before the 24 completed week of gestation, and included 62 cases of spontaneous miscarriage, 7 ectopic pregnancies, and 3 molar pregnancies (Table 13). The spontaneous miscarriage group included 10 symptomatic pregnancies (threatened bleeding with or without abdominal pain), 10 asymptomatic pregnancies (no signs of threatened miscarriage), 26 missed abortions (20 non-viable embryonic+ 6 blighted ova or anembryonic pregnancy), 11 incomplete miscarriages, and 5 inevitable miscarriages.

Types of pregnancy failure	N	% of group 2 N=72	% of total study group N=289
All spontaneous miscarriages	62	86	21
Threatened	10	13.8	3.4
Non-threatened	10	13.8	3.4
Missed	26	36.1	8.9
Incomplete	11	15.2	3.8
Inevitable	5	6.9	1.7
Ectopic	7	9.7	2.4
Molar	3	4.1	1

Table 13 Incidence of pregnancy failures at ≤24 weeks of gestation in the "non-continuing group" (Group 2).

The distribution of serum and urinary levels for all hCG analytes in the "continuing group" (Group 1) and the "non-continuing" group (Group 2) are shown in Figures 28-33. Levels were unevenly distributed, and are therefore log-transformed for easy viewing and comparison.

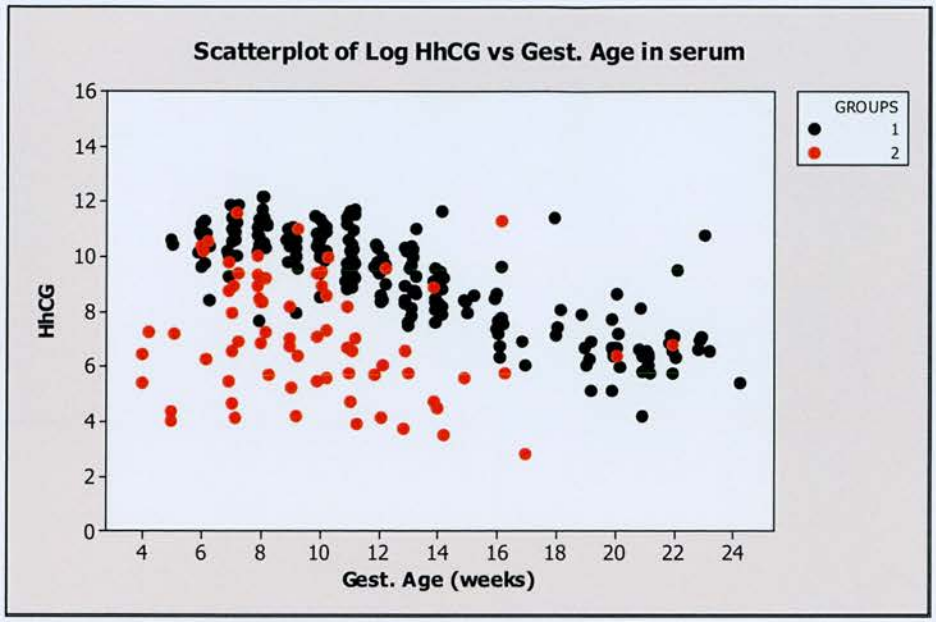

Figure 28 Log data of HhCG in serum in Groups 1 and 2. [Group 1, continuing group; Group 2, non-continuing group.]

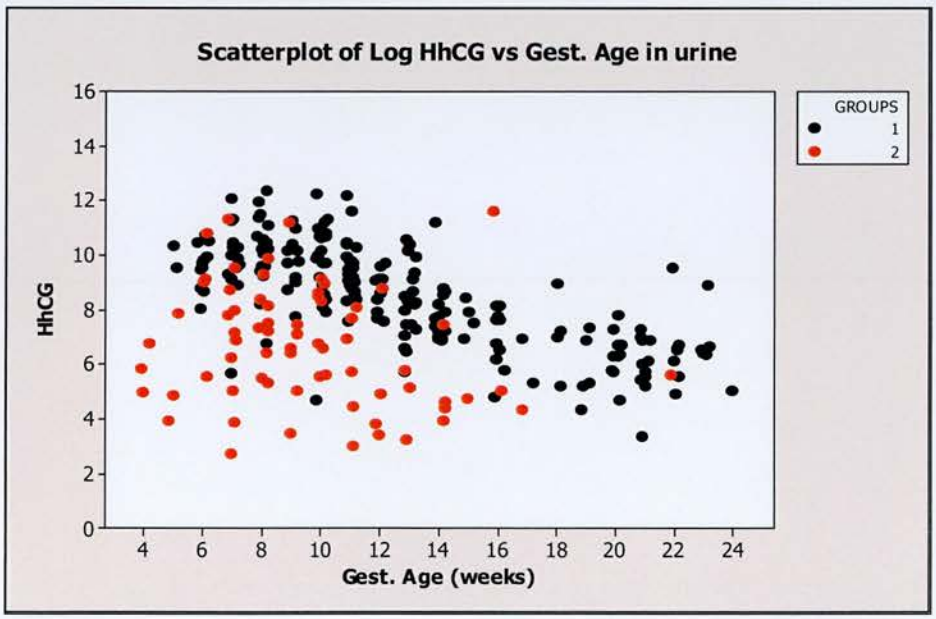

Figure 29 Log data of HhCG in urine in Groups 1 and 2. [Group 1, continuing group; Group 2, non-continuing group.]

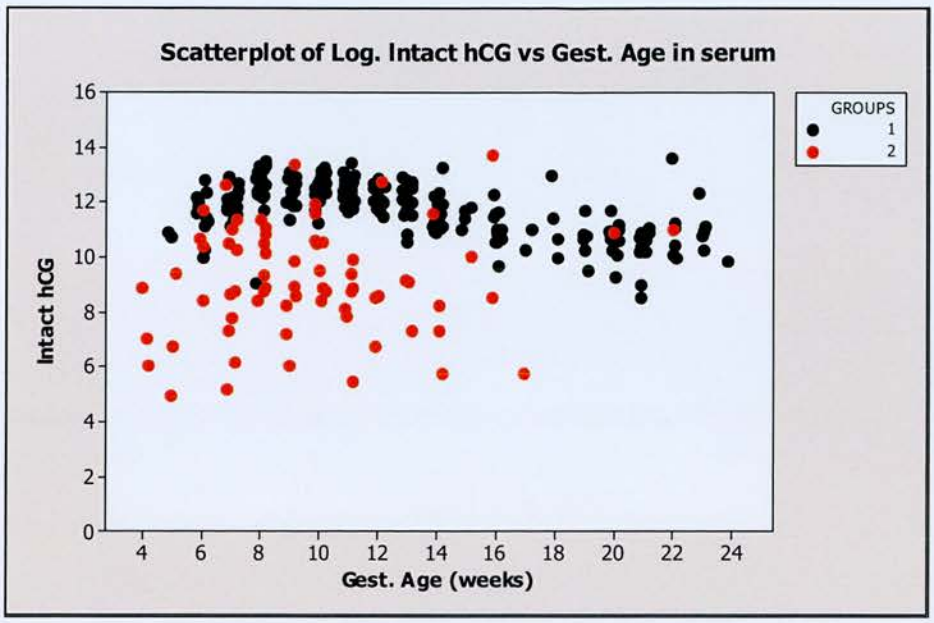

Figure 30 Log data of hCG in serum in Groups 1 and 2. [Group 1, continuing group; Group 2, non-continuing group.]

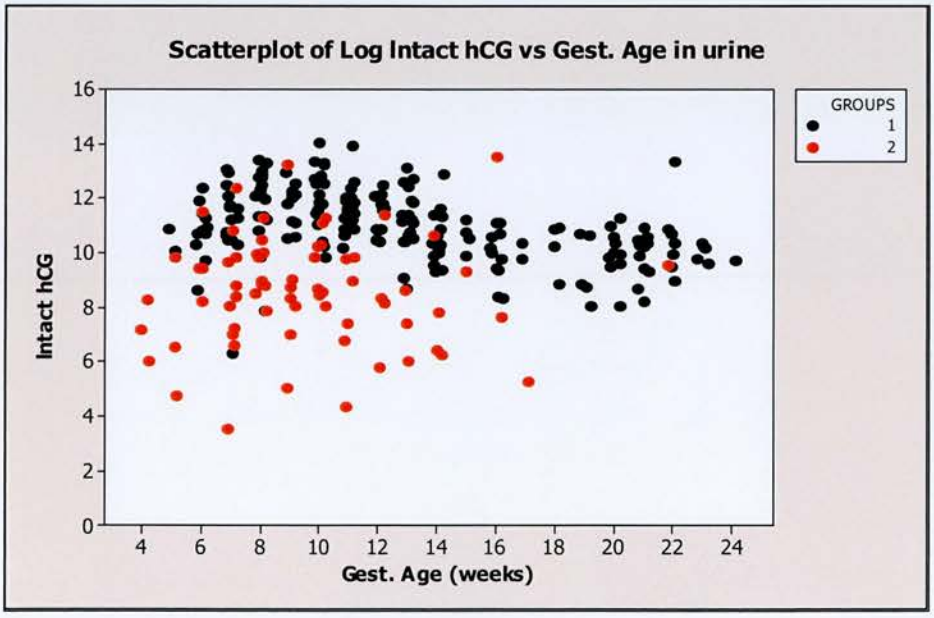

Figure 31 Log data of hCG levels in urine in Groups 1 and 2. [Group 1, continuing group; Group 2, non-continuing group.]

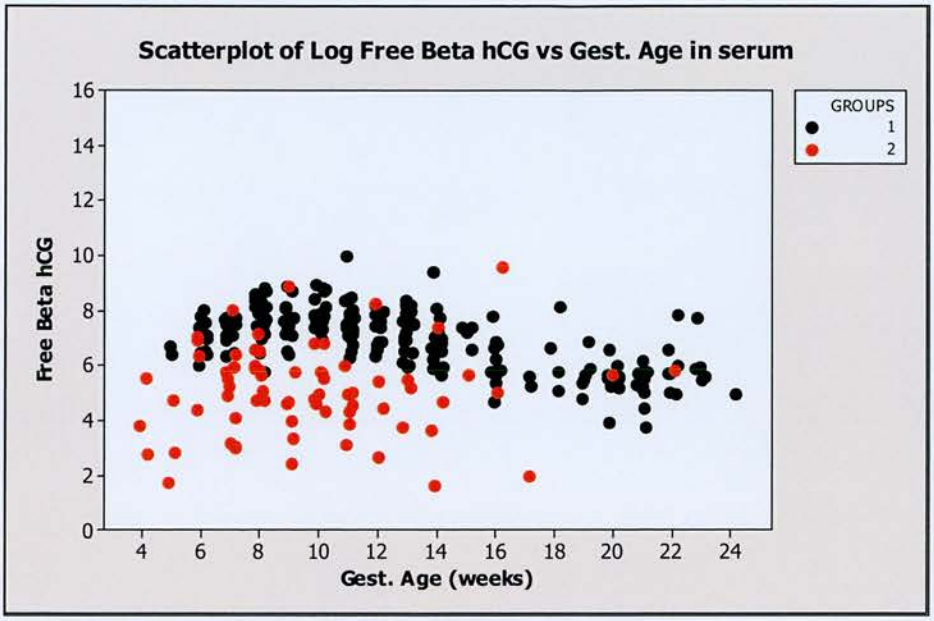

Figure 32 Log data of hCGβ levels in the serum in Groups 1 and 2. [Group 1, continuing group; Group 2, non-continuing group.]

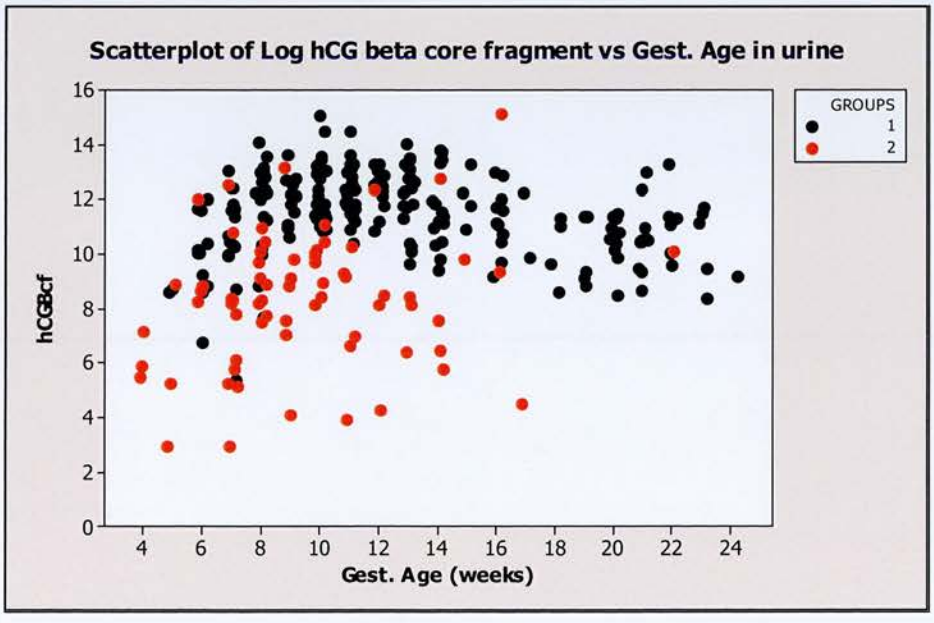

Figure 33 Log data of hCGβcf in urine in Groups 1 and 2. [Group 1, continuing group; Group 2, non-continuing group.]

		Group 1		Group 2		95% CI for difference	P-value
		N	Median	N	Median		
Serum	hCG	217	159440	71	7477	(96614, 165365)	<0.001
	hCGβ	217	1346.8	71	153.3	(654.2, 1310)	<0.001
	HhCG	217	15785	71	970	(4526, 16092)	<0.001
Urine	hCG	211	58727	69	5610	(32728, 63109)	<0.001
	hCGβcf	211	109116	69	4474	(64582, 122315)	<0.001
	HhCG	211	5941.2	69	741.8	(1610.8, 6239.3)	<0.001

Table 14 Median concentrations of hCG molecular forms with inter-quartile levels and the differences in Group 1 vs. Group 2, both in serum and urine.

Table 14 shows median levels of HhCG and other hCG related molecules in both groups while Figure 34 shows the box-plots for all hCG concentrations in Groups 1 and 2 (continuing group vs. miscarriage group). There is a statistically significant difference between Groups 1 and 2 with Group 1 having higher results for each of the hCG molecular forms, in both serum and urine.

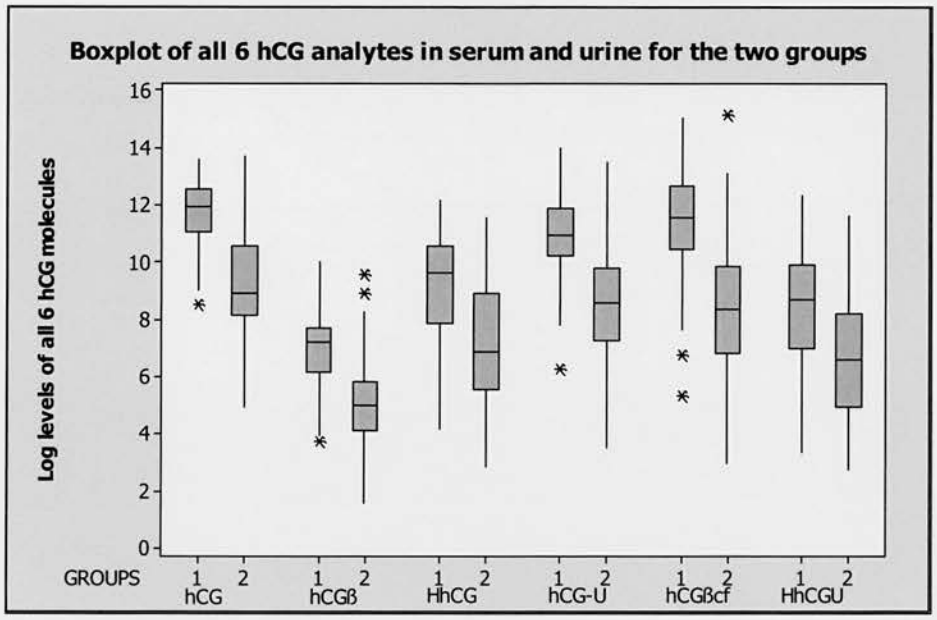

Figure 34 Box-plots of all hCG concentrations in Groups 1 and 2, comparing raw data irrespective of gestational age. [Median, inter-quartile range (box) and 10^{th} to 90^{th} centiles (whiskers) plotted. All asterisks indicate values outwith the whiskers.]

It is evident from the above that all six hormonal parameters investigated were higher in the "continuing group" than in the "non-continuing group" ($p< 0.001$ for all analytes) (Table 14, Figure 34).

Median concentrations of HhCG in Groups 1 and 2 in serum were 15785 pmol/L and 970 pmol/L (95% CI 4526, 16092), respectively, whereas in urine the levels were 5941.2 pmol/L and 741.8 pmol/L (95% CI 1610.8, 6239.3), respectively. This corresponds to 94% and 88% reduction in serum and urine HhCG levels, respectively. Similar lower median levels in Group 2 were observed for the remaining 4 hCG forms, when compared with Group 1. Serum hCG and hCGβ showed a 93.4% and 88.7% reduction, whereas urinary hCG and hCGβcf showed 90.5% and 96% reduction in their hormonal levels during pregnancy failures. Of all types of pregnancy failures, ectopic pregnancy had the lowest levels for all hCG forms, where

as the highest levels were seen in molar pregnancy (Figures 35-36). However, no significant statistical difference was found between the Group 2 miscarriage-subtypes.

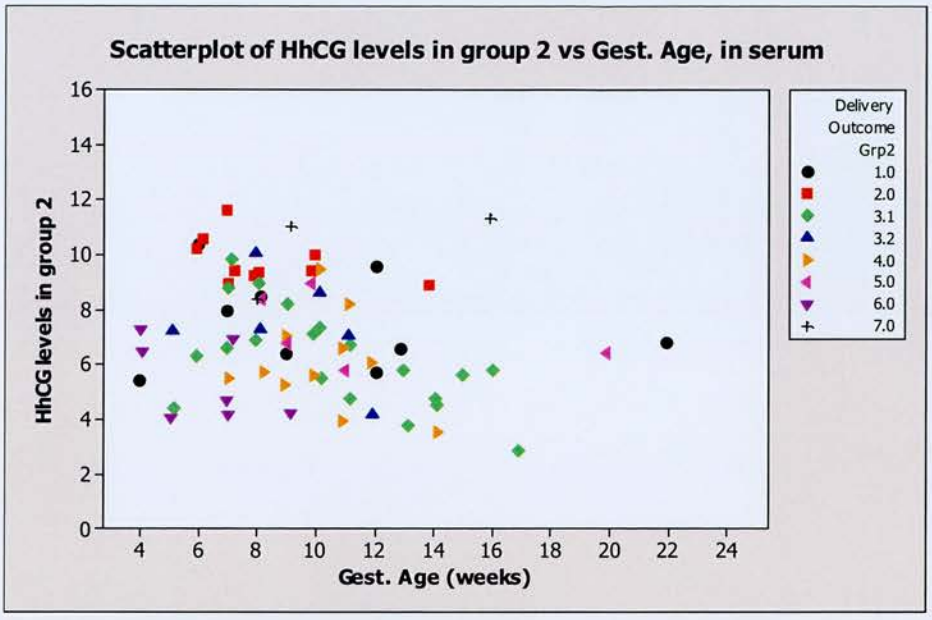

Figure 35 HhCG Serum concentrations (log data) in various types of pregnancy failures amongst the non-continuing group. [Threatened ●; non-threatened ■; missed ◆; blighted ovum ▲; incomplete ◢; inevitable ◢; ectopic ◢; molar +.]

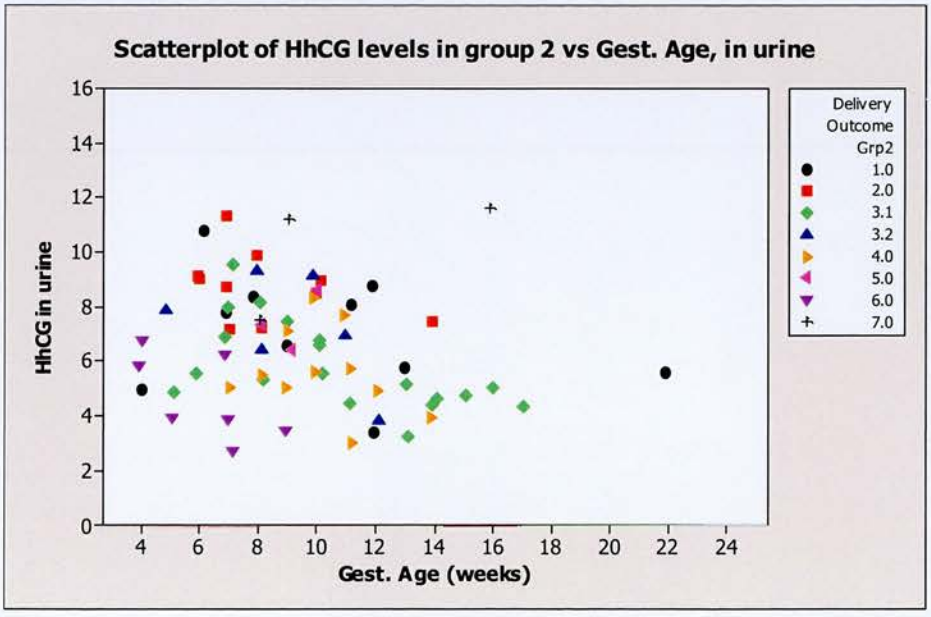

Figure 36 Urinary HhCG concentrations (log data) in various types of pregnancy failures amongst the non-continuing group. [Threatened ●; non-threatened ■; missed ◆; blighted ovum ▲; incomplete ◢; inevitable ◢; ectopic ◢; molar +.]

In contrast to individual hormone levels, the ratios of HhCG/ hCG (serum and urine), HhCG/hCGβ, hCGβcf, and serum HhCG/(hCG+ βhCG) remained unchanged in

Groups 1 and 2. (Figures 37-41). The variability in both groups reduced as the gestational age advanced with no statistical difference between the variability of the two groups.

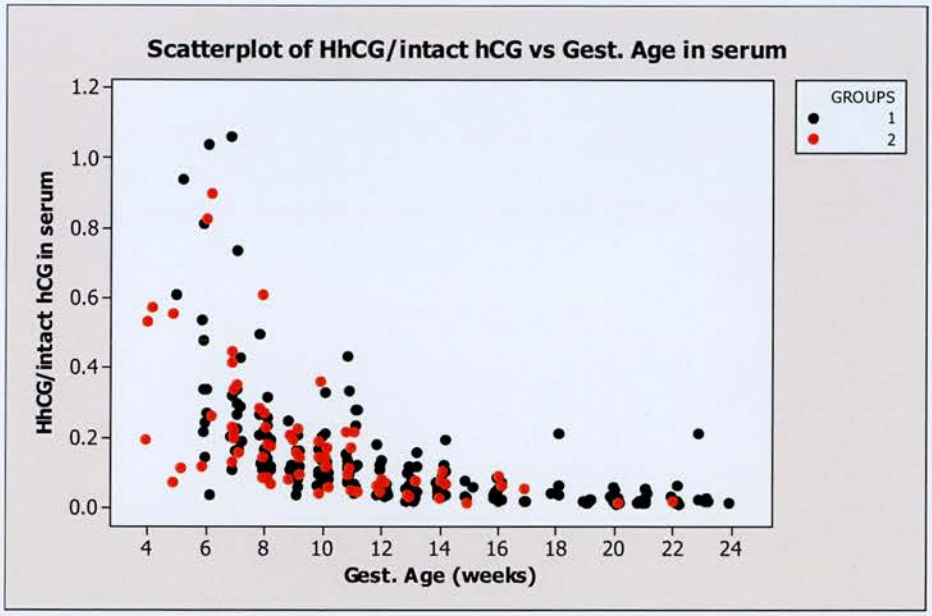

Figure 37 Ratio of HhCG/hCG in serum. P-value is NS in Group 1 vs. Group 2. [Group 1, continuing group; Group 2, non-continuing group.]

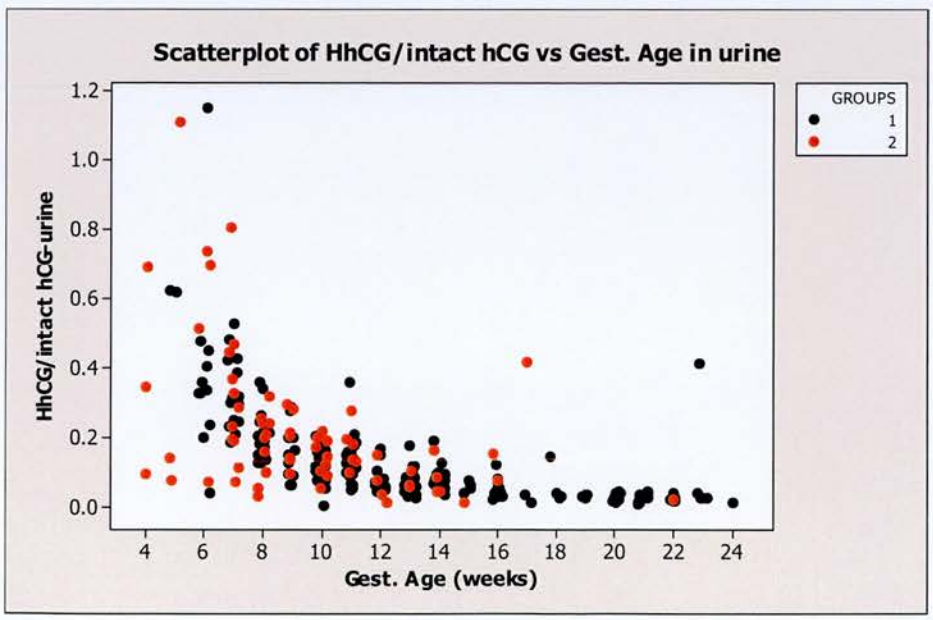

Figure 38 Ratio of HhCG/hCG in urine, P-value is NS in Group 1 vs. Group 2. [Group 1, continuing group; Group 2, non-continuing group.]

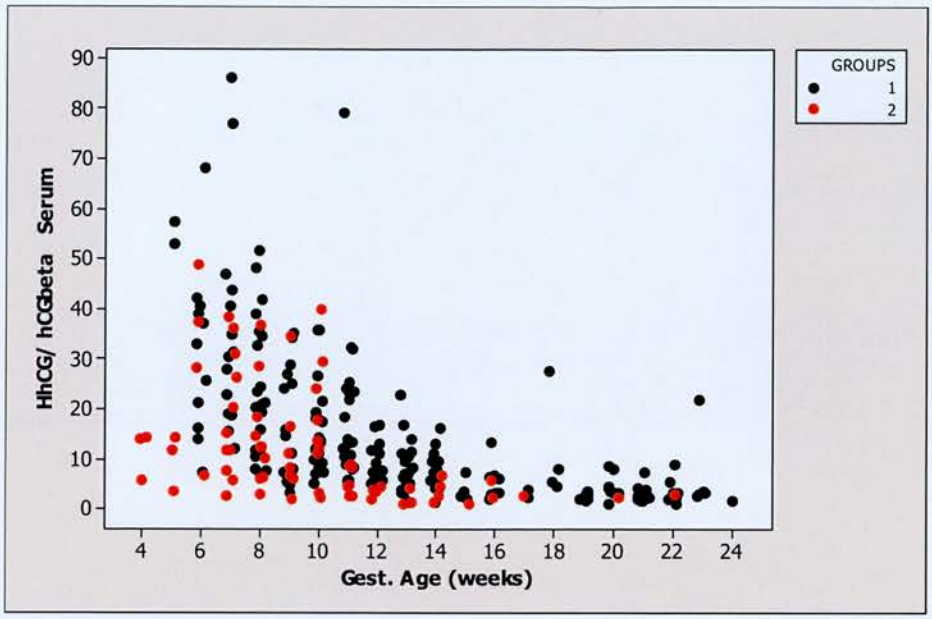

Figure 39. Ratio of HhCG/hCGβ in serum, P-value is NS in Group 1 vs. Group 2. [Group 1, continuing group; Group 2, non-continuing group.]

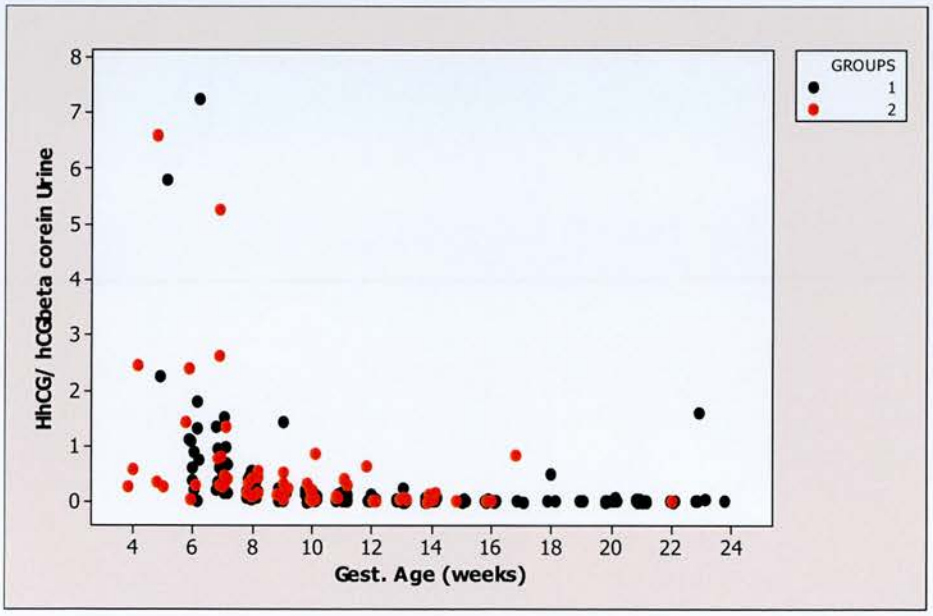

Figure 40 Ratio of HhCG/hCGβcf in urine, P-value is NS in Group 1 vs. Group 2. [Group 1, continuing group; Group 2, non-continuing group.]

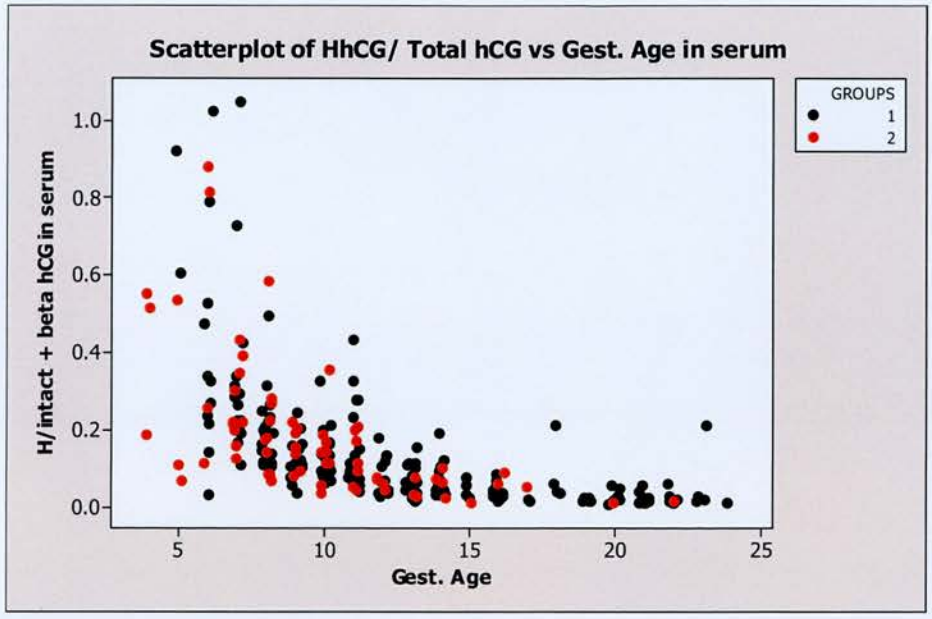

Figure 41 Ratio of HhCG/(hCG+ hCGβ/total hCG) in serum, P-value is NS in Group 1 vs. Group 2. [Group 1, continuing group; Group 2, non-continuing group.]

3.4.4 Comparison of patients with threatened bleeding in Groups 1 and 2

A total of 18 women whose pregnancy continued past 24 weeks had threatened bleeding (n=17 on or before 14 weeks, n=1 at 22 weeks, total=18) and were compared with 10 pregnancies presenting with threatened bleeding (n=9 ≥14 weeks and 1 at 22 weeks) but who eventually spontaneously miscarried at or before 24 weeks of gestation (Table 15).

		Group 1		Group 2		95% CI for difference	P-value
		N	Median	N	Median		
Serum	hCG	18	228558	9	9381	(51614, 270789)	0.003
	hCGβ	18	2021.7	9	320.3	(703.9, 2183.0)	0.003
	HhCG	18	40018	9	937	(13154, 48600)	0.001
Urine	hCG	16	80410	10	10818	(31323, 191903)	0.005
	hCGβcf	16	69124	10	9074	(1005, 154294)	0.048
	HhCG	16	17318	10	1602	(1966, 41185)	0.007

Table 15 Median results (95% CI) of all hCG forms showing statistically significant differences between Group 1 (threatened-continuing) and Group 2 (threatened-miscarriage).

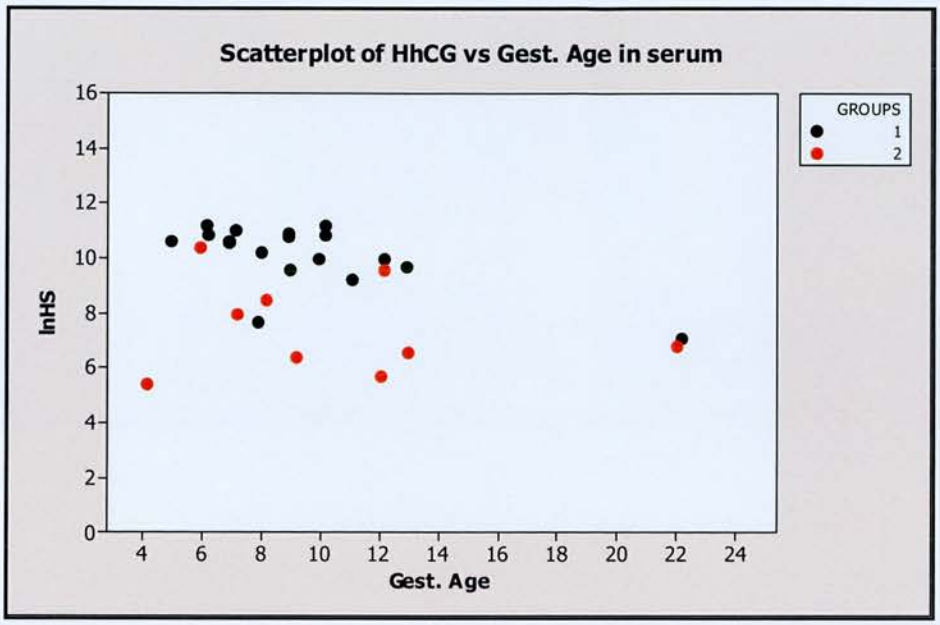

Figure 42 Log (HhCG) levels in serum in Group 1 (threatened-continuing group) (●),and Group 2 (threatened-miscarriage group) (●).[Group 1, continuing group; Group 2, non-continuing group.]

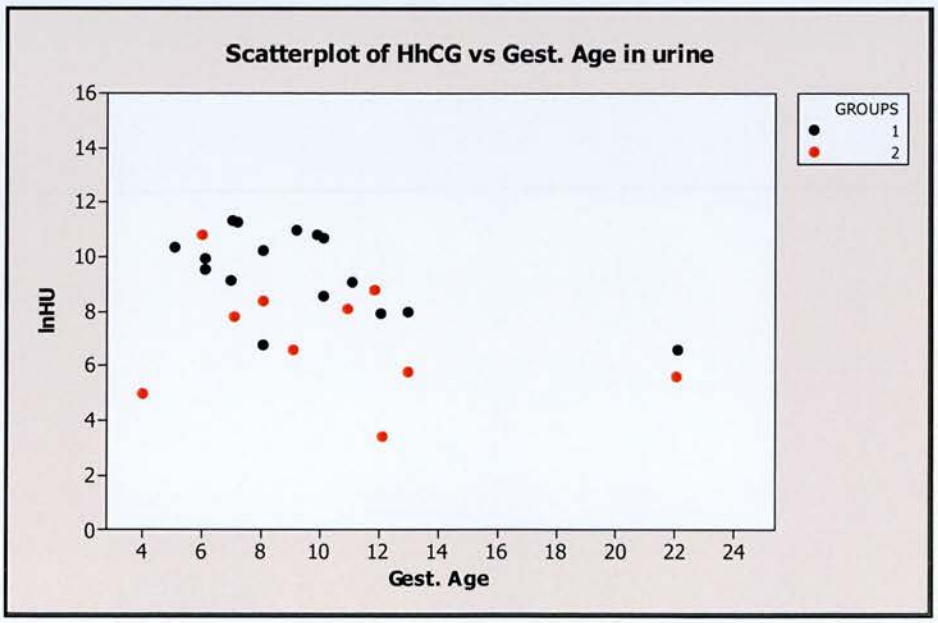

Figure 43 Log HhCG levels in urine in Group 1 (threatened-continuing group), (●) and Group 2 (threatened-miscarriage group) (●).[Group 1, continuing group; Group 2, non-continuing group.]

As seen in Figures 42 and 43 and Table 15, HhCG, and other hCG forms shows higher levels in pregnancies continuing to term than those which eventually

spontaneously miscarried, indicating that lower levels of hCG hormones were only found in pregnancies destined to fail, irrespective of the symptoms of threatened bleeding.

3.4.5 Embryonic and non-embryonic miscarriages

Of eighteen women with 1st or 2nd trimester bleeding in this study population who eventually continued their pregnancy beyond 24 weeks of gestation (threatened-continuing pregnancy), only 72% (n=13) had an uneventful pregnancy outcome. The remaining 28% were associated with a combination of complications such as PIH, gestational diabetes, spontaneous preterm deliveries and intrauterine fetal demise.

Both embryonic (gestational sac with dead embryo, n=20) and anembryonic (empty sac, n=6) missed miscarriages presented with lower maternal and serum concentrations of HhCG and other hCG-related forms when compared to normal uneventful pregnancy ($P < 0.001$). When we investigated the effect of the presence or absence of an embryo in missed abortion on placental hormonal levels, we found medians of all 6 hormones were higher in anembryonic, (also known as blighted ovum), than embryonic missed miscarriages (Table 16). However the differences were not statistically significant because of the wide range of concentrations.

hCG analytes	Miscarriage type		Mean (pmol/L)	Median (pmol/L)	25th–75th centiles	p value
SERUM	HhCG	Embryonic	2186	431	115- 1479	0.22
		Anembryonic	5472	1408	862- 9897	
	hCG	Embryonic	14863	6839	2876- 17877	0.33
		Anembryonic	26057	12328	4983- 50106	
	hCGβ	Embryonic	167	102.8	51.1- 226	0.22
		Anembryonic	326	128	89.9- 545	
URINE	HhCG	Embryonic	1382	202	101- 963	0.13
		Anembryonic	4049	1807	471- 9659	
	hCG	Embryonic	7541	4244	872- 7621	0.07
		Anembryonic	21303	13226	2008- 41541	
	hCGβcf	Embryonic	18602	3456	1090- 11389	0.24
		Anembryonic	6695	8457	3113- 34789	

Table 16 Median concentrations with interquartile ranges of all six hCG analytes. Differences not statistically significant [p>0.05, Mann-Whitney test]

3.4.6 HhCG levels in uneventful pregnancy vs. late pregnancy complications

The "continuing group" consisted of 217 pregnancies, of which 137 were uneventful pregnancies (i.e. term deliveries with no complication), constituting 63% of Group 1 and 47.4% of the total study population. The remaining 37% had adverse pregnancy complications, either as an isolated condition or in combination with other complications. Table 10 shows outcome variables of the "continuing group" and their incidences in study population.

There were 18 patients in the continuing group who had threatened bleeding during the first trimester but who continued their pregnancy until term. There was no statistically significant difference between these 18 pregnancies compared to the 137 pregnancies with an uneventful outcome. However, all hCG levels were significantly higher in those women compared to the miscarriages group in Group 2, indicating that irrespective of bleeding during first trimester, pregnancies destined to progress to term have normal levels of all hCG forms.

Other outcome measures in this group included: pregnancy complications (Table 10): pregnancy induced hypertension (PIH), with or without proteinuria (n=14), small for gestational age (SGA) n=11, spontaneous preterm (n= 8), gestational diabetes (n=14), and intrauterine fetal death /neonatal death (n=8). The non-proteinuric PIH included 10 patients, whereas the proteinuric hypertension included pre-eclampsia (n= 3) and eclampsia (n=1). As seen in Figures 44 and 45, there was no statistically significant difference in the levels of HhCG in urine or serum between any of the late pregnancy complications and the uneventful pregnancy group, indicating that HhCG is a poor indicator of pregnancy adverse outcome in later gestation. Similar results were observed for the remaining hCG forms in serum and urine.

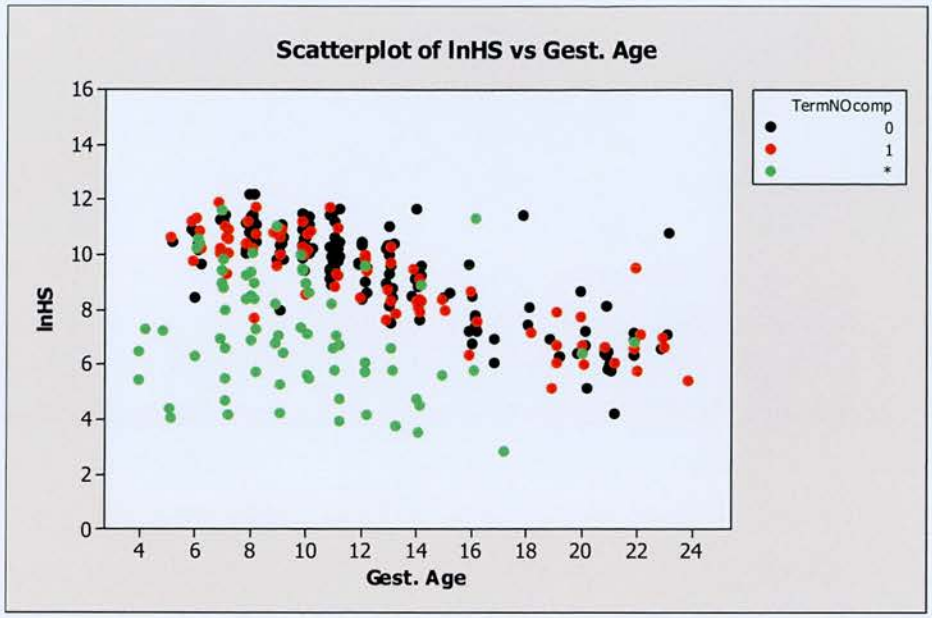

Figure 44 Serum HhCG levels in uneventful pregnancy (●) vs. continuing pregnancy with late obstetrics complications (●). Green dots (●) represent HhCG levels in the non-continuing group which clearly shows significant lower levels when compared to the previous two. [InHS, HhCG in serum; TermNOcomp, continuing pregnancy with (1) or without (0) complications; asterisks indicate non-continuing pregnancy.]

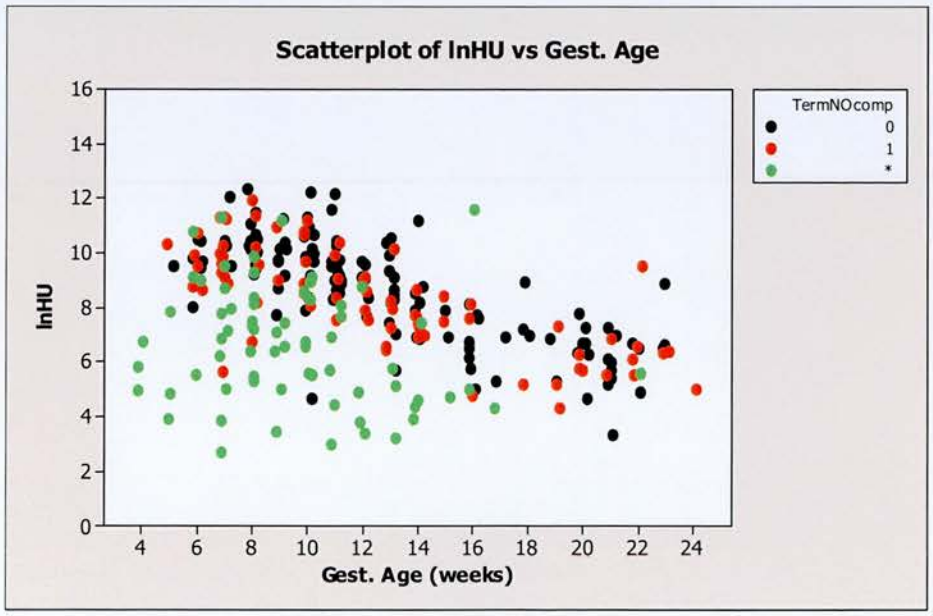

Figure 45 Urinary HhCG levels in uneventful pregnancy (●) vs. continuing pregnancy with late obstetrics complications (●). Green dots (●) represent HhCG levels in the non-continuing group, which clearly shows significantly lower levels when compared to the previous two. [InHU, HhCG in urine; TermNOcomp, continuing pregnancy with (1) or without (0) complications; asterisks indicate non-continuing pregnancy.]

In summary, this study has demonstrated that maternal serum and urinary HhCG levels between 6-24 weeks were lower in pregnancies resulting in miscarriages, but no difference in the levels of HhCG was found in pregnancies continuing beyond 24 completed weeks, and in pregnancies that subsequently developed complications such as PIH, gestational diabetes and growth restrictions, indicating that HhCG may be a useful predictor for detecting pregnancy failures during the first and second trimesters but not of any other adverse outcome occurring later in gestation. To test this hypothesis, HhCG was tested by a ROC analysis for its diagnostic efficacy in predicting pregnancy failures.

3.5 ROC analysis

By calculating specificity and sensitivity, ROC curves were constructed for the six hCG analytes measured in serum and urine to establish their diagnostic accuracy in predicting adverse pregnancy outcome (Figure 46 and Table 17).

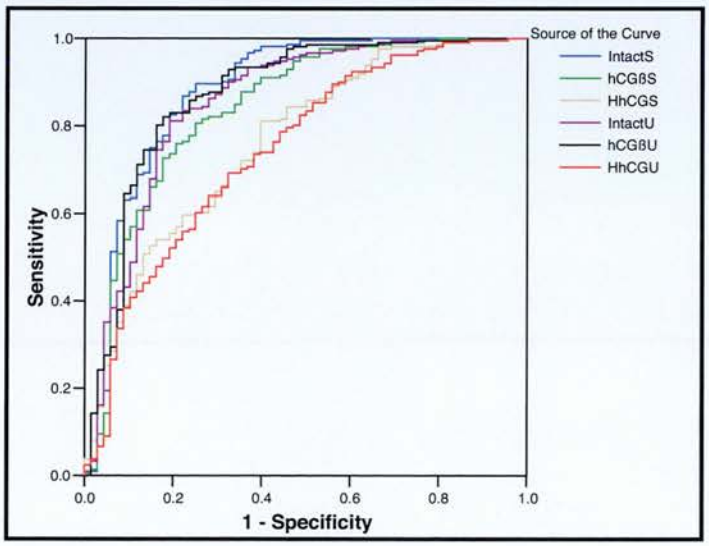

Figure 46 ROC curve analysis for hCG parameters to determine whether women will continue their pregnancy beyond 24 weeks.

	hCG variables	AUC	Std. Error	95% CI	DA
Serum	hCG	0.880	.029	0.824-0.937	+
	hCGß	0.841	.031	0.780-0.901	+
	HhCG	0.764	.034	0.698-0.831	±
Urine	hCG	0.855	.030	0.795-0.914	+
	hCGßcf	0.873	.029	0.817-0.929	+
	HhCG	0.744	.036	0.675-0.814	±

Table 17 Comparison of hCG molecular forms by ROC analysis. [AUC, area under the curve; DA, diagnostic accuracy; +, good and ± moderate DA.]

The molar concentrations of the six hCG molecular forms were used to ROC curves and the area under the curves were calculated to assess the diagnostic accuracy of these analytes. ROC curves represent the full spectrum of possible sensitivity-specificity pairs evaluating a test for possible clinical application, with the y-axis representing sensitivity (true positive) and the x-axis representing 1-specificity (true negative).

3.5.1 ROC analysis for miscarriages

The model created by Duc et al. [217] to rank the accuracy of a diagnostic test was used. AUC values of 0.91-1.00 and 0.0-0.09 are rated as excellent (++), 0.81-0.90 and 0.10-0.19 as good (+), 0.61-0.80 and 0.20-0.39 as moderate (±), and 0.40-0.60 as poor accuracy (-) [217]. AUCs for HhCG in serum and urine were 0.764 ± 034 and 0.744 ± 036 respectively for the group of pregnant women who continued beyond 24 weeks of gestation vs. the group who miscarried before completed 24 weeks of gestation and shows moderate accuracy. However the diagnostic accuracy of hCG (in both serum and urine), hCGβ and hCGβcf were better than HhCG, as the AUC showed good diagnostic accuracy (0.81-0.90) for the remaining analytes when compared with HhCG. HCG in serum had the best AUC (0.880) compared to the other hCG analytes (Figure 46 and Table 17).

The sensitivity represents all patients who are correctly identified as a continuing pregnancy group, with the specificity representing all pregnancy failures correctly identified. Hence it is important for a screening test to have a high specificity and negative predictive value, but without compromising the sensitivity. False-negative results must be avoided though a moderate number of false-positive results can be considered as acceptable. When the optimal specificity cut-off value was chosen to maximize detection of 70% of all pregnancy failures, a HhCG level of 4846 pmol/L (~ 200 μg/L) had a sensitivity of 66% (i.e. 66% detection of a "continuing pregnancy", or a positive predictive value) in serum . In urine, the same cut-off value of 200 μg/L yielded better detection of failures with a specificity of 78% but a lower sensitivity of 55%. Using the same cut-off value in serum, 100% of ectopic pregnancies could be detected but at the expense of very low sensitivity (33%), showing very high negative predictive value to correctly identify miscarriages.

3.5.2 ROC analysis for ectopic pregnancy

The ability of HhCG to discriminate ectopic pregnancy (n= 7) from spontaneous miscarriages which include threatened miscarriages, missed abortion, blighted ovum, incomplete or an inevitable miscarriage (n= 61, one n missing) was tested under the ROC curve (Figure 47), the AUC was 0.74 which according to model created by Duc et al. [217] is ranked as moderately accurate diagnostic test (±) (Table 18). However wide confidence interval, together with high standards of error obtained due to the small number of samples, makes the findings of HhCG performance under the ROC curve less reliable.

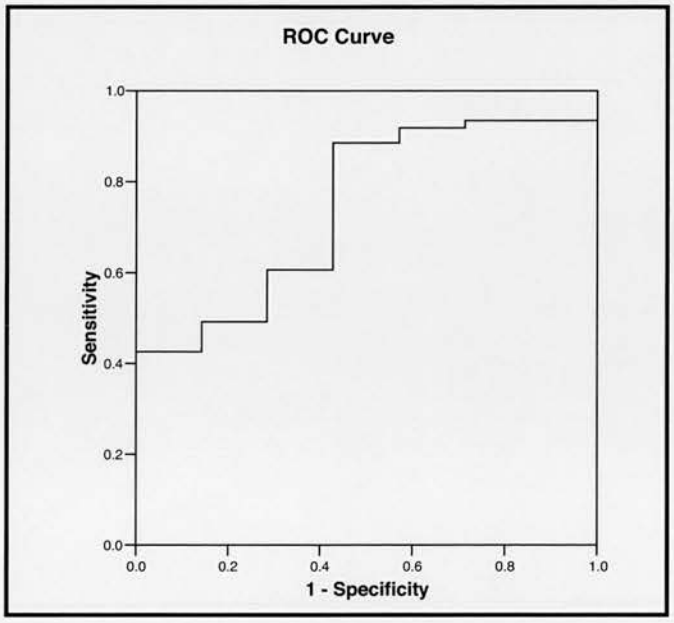

Figure 47 ROC curve analysis for hCG in discriminating ectopic pregnancy from other types of miscarriages.

		Asymptotic 95% Confidence Interval	
Area	Std. Error	Lower Bound	Upper Bound
.742	.088	.570	.915

Table 18 Area under the curve (AUC) showing the moderate diagnostic accuracy of HhCG in serum with wide confidence interval.

3.6 Comparison of HhCG results from two methods

The relationship between levels of HhCG produced in two different immunoassay system (DELFIA vs. Quest) was tested using "Bland and Altman plots" that plot the difference between paired results (calculated as DELFIA minus Quest HhCG result) vs. the mean of the paired results.

The Bland and Altman plots (Figures 48 and 49) indicate good qualitative agreement between the methods, as there is relatively little scatter of results, but very poor quantitative agreement. However, inspection clearly indicates that for both serum and urine the line of equivalence between the methods has a significant slope but passes through zero. This may well be accounted for by differences in calibration between the methods. There is no International Standard for HhCG so assays are calibrated against different standards, which is likely to contribute to the differences observed.

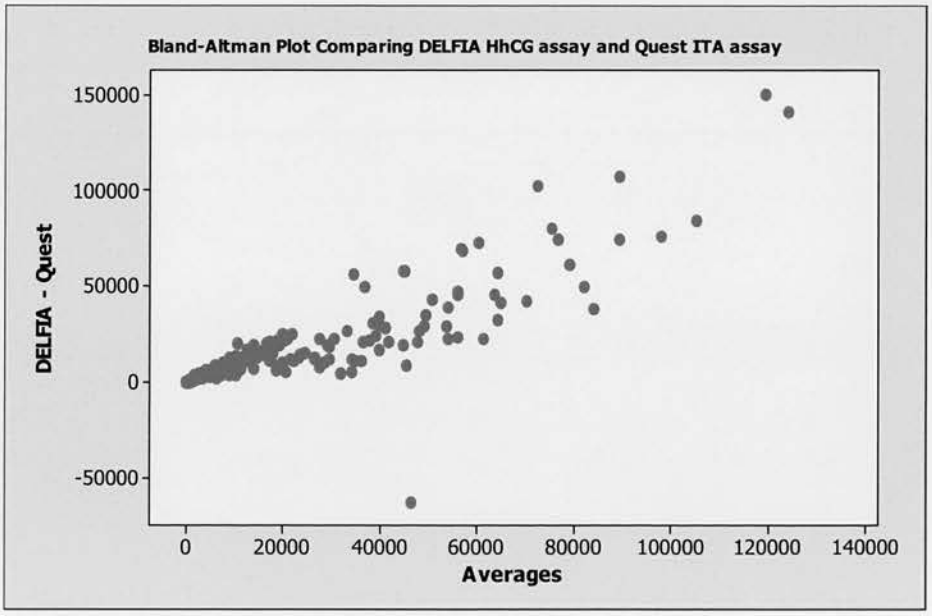

Figure 48 Bland-Altman plot comparing serum HhCG results obtained in the Perkin-Elmer DELFIA method and the Quest Diagnostics method.

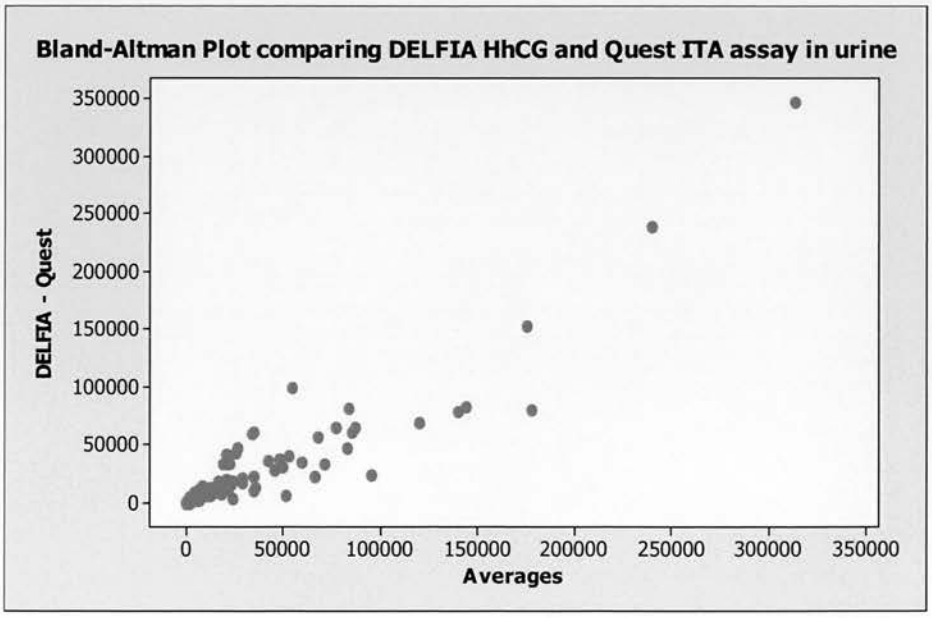

Figure 49 Bland-Altman plot comparing urine HhCG results obtained in the Perkin-Elmer DELFIA method and the Quest Diagnostics method.

3.7 Discussion of results for the UAE cohort

3.7.1 Early pregnancy

3.7.1.1 Uneventful pregnancy and spontaneous miscarriage

In normal pregnancy, the cytotrophoblasts are the dominant placental cells during early gestation, producing HhCG. The syncytiotrophoblast (syncytiotrophoblast) (differentiated trophoblast transformed from cytotrophoblast) becomes dominant later in gestation, and produces hCG [70, 92, 250]; [69, 70, 97, 244]. The rate of differentiation of cytotrophoblast into syncytiotrophoblast appears to be the main factor that leads to the synthesis of hCG and its subunits by the syncytiotrophoblast [92, 93, 106]. The production these molecular forms of hCG is under stringent physiological control, and is reported to be altered under different pathological conditions. Early pregnancy events such as defective implantation or placentation have been linked to the development of various complications and diseases during pregnancy including miscarriages, pre-eclampsia, FGR, leading to possible alterations in placenta hormonal parameters.

The aim of this prospective observational cohort study was to explore the levels of HhCG in serum and urine, along with other 3 hCG molecular forms: hCG, hCGβ, and hCG βcf, in normal pregnancy and pregnancy-related disorders, and assess their diagnostic ability in predicting an adverse pregnancy outcome.

Analysis of matched serum-urine samples from the 1st and 2nd trimester pregnancy samples in the 'pregnancy-continuing' group, revealed higher urinary and serum HhCG levels during the 1st trimester compared to the 2nd trimester (Table 11, Figures 24 and 25). This is in agreement with previous studies of normal pregnancies, conceived naturally or through IVF, showing high levels of HhCG in serum and urine during early pregnancy, with peak concentrations achieved during the first six weeks of gestation, then rapidly declining and remaining at lower levels as pregnancy advances [67, 244, 251]. The decrease of HhCG with advancing gestation is secondary to the simultaneous decline in the number of differentiating cytotrophoblast cells [70].

Throughout pregnancy there are changes in the immunoreactivity of total hCG, first as a result of production of hormone initially by cytotrophoblasts, then shifting gradually to the syncytiotrophoblasts [70, 92, 93, 145, 222, 252]. Thus, HhCG during early pregnancy is replaced by hCG molecular forms characteristic of later pregnancy, which remain the predominant form for the remainder of the pregnancy. Studies have

shown that HhCG accounts for >80%, 63% and 50% of the total hCG concentration in the first, second and third weeks following implantation (i.e. the 3rd to 5th completed week of pregnancy), respectively. The proportion of HhCG decreases during the remainder of the first trimester (<25%) and declines much further through the 2nd and the 3rd trimesters of pregnancy, and by then constituting less than 2% of the total hCG immunoreactivity. The findings in this study are comparable to previous studies showing that reported median proportions of serum HhCG (11%) during the first trimester, (i.e. 6th -12th weeks of gestation), compared to 3% during the second trimester, in the pregnancy-continuing group. Although 3rd trimester samples were not included in this study, others have reported a median of 1.7 % HhCG molecules during the 27th–41st completed week of pregnancy (Table 19) [251].

Gestational age	Median (%)	Range
3 weeks + 6 days	92	55-100
4 weeks + 6 days	73	15-100
5 weeks + 6 days	50	10-100
6 weeks + 6 days	26	8-53
7 weeks + 6 days	14	2-21
2nd trimester	2	0-31
3rd trimester	2	0-4

Table 19 The proportion of total hCG immunoreactivity in urine samples due to HhCG throughout pregnancy. [253]

Also in this study it was also noted that the proportions of HhCG to other hCG molecular forms [i.e. HhCG/hCG, HhCG/(hCG+ hCGβ), HhCG/hCGβ], were all statistically different from the 1st trimester to 2nd trimester (i.e. fell significantly between the 1st to 2nd trimester) (Table 12). However the molar ratios of hCG, hCGβ, and hCG βcf, remained unchanged, probably indicating that the production of hCG and its related molecules, are regulated differently to the HhCG (which is produced by the cytotrophoblast and which is significantly reduced following the its differentiation into syncytiotrophoblast. Altered ratios may reflect the level of differentiation and hyperplasia of the trophoblast and may help differentiate normal pregnancies from complicated ones and invasive trophoblastic diseases from the non-invasive ones [75, 207, 254, 255].

It was not possible to show in this study very high proportions of HhCG, as obtained in other studies [66, 130] during early gestations, since the UAE study population consisted of spontaneous conceptions presenting at more than 6 weeks of gestation, (mean gestational age of 9 weeks), at the time of sampling during the first trimester,

and at 17 weeks in the second trimester. However, the pattern of HhCG production observed in this study confirms that the glycosylation profile of hCG changes as gestation progresses [25, 26, 50, 66, 130]. The serum levels of hCG and hCGβ, along with urinary levels of hCG and hCGβcf followed a similar pattern of high concentrations during the 1st trimester compared to the 2nd trimester (Figure 27-28, Table 16). The observed decrease in hormonal concentrations as pregnancy progresses are in concordance with several previous publications [95, 113, 115, 133, 134, 256, 257].

During normal pregnancy, serum hCG concentrations increase at an exponential rate, reaching peak concentrations of approximately 100000 U/L (in relation to 1st IRP) (~290000 pmol/L in relation to the 3rd IS, 75/537) between the 7th and 10th weeks of gestation, and decrease 10-fold thereafter, before reaching a plateau of approximately 36000 U/L (~104,400 pmol/L) at the beginning of the 2nd trimester and remaining at a relatively constant level until nearer term, when there is a small increase before delivery [94, 95, 207].

The molar ratio of hCGβ to total hCG (hCGβ/hCGβ+ hCG) ranged from 0.3% to 3% in serum samples of 6-24 weeks of gestation with no statistical difference found in the molar ratios from the 1st to 2nd trimester (Table 12). This was similar to the range reported in literature (i.e. 0.1% to 3.6%). Higher ratios were observed in trophoblastic cancers and invasive neoplasms, and hCGβ is thought to be useful in differentiating malignant trophoblastic tumors from benign ones [75, 115, 207, 255, 257-259].

The major immunoreactivity in urine is due to hCGβcf, the terminal degradation product of hCGβ, which comprises 50%-100% of total hCG urinary immunoreactivity. hCGβcf can be detected in serum in quantities as low as 0.3% of the hCG content but the concentrations in urine are ~4000-fold greater than those in serum [114-116]. In this study, the levels of hCGβcf in the 1st trimester of the continuing-pregnancy group, was 223,218 pmol/L, and fell to 75,677 pmol/L during the second trimester. The decrease in median concentrations was concurrent with the reduction in the production of hCG and hCGβ with advancing gestation. It has been shown that hCGβcf concentrations are initially lower than hCG concentrations, increasing sharply from 5 weeks of gestation, so that they equal hCG concentrations (mol/mol) at 6-7 weeks of gestation, exceeding hCG concentrations thereafter. In this study, the median proportion of hCGβcf to hCG (hCG βcf/hCG on a molar basis) during the first trimester was 60% (25th -75th centiles 49%-72%) and during the second was 68% (25th -75th centiles 60%-79%), showing no statistically significant difference between the

two trimesters (by student's *t* test for paired samples), and indicating that the pattern of production of both hormones in relation to one another remains constant (Table 12). These ratios are said to be altered in trophoblastic and non-trophoblastic neoplasms such as cervical and pancreatic cancers [116, 205, 258].

Many "sandwich assays" used in recent days include multiple antibodies raised to different sites on hCG and its free subunits, often using one monoclonal antibody to capture hCG through a specific site which is then detected by separate monoclonal or polyclonal antibodies raised against a distant site on the hormone. Due to differences in the combinations of antibodies used, hCG and its free subunits are detected variably by different immunoassays [115, 197]. However, to detect the total hCG immunoreactivity in serum, an assay should ideally be able to recognize hCG + hCGn + HhCG + hCGβ + hCGβn [115, 208, 212, 260]. Given the specificities of the assays used in this study, which detect both the nicked- and non-nicked forms of hCG and hCG β, together with the 100% cross-reactivity with HhCG of the hCG assay used in this study (Stenman et al., unpublished data), total hCG immunoreactivity in serum was calculated as (hCG + hCGβ). In urine however, this was not possible because hCGβ was not measured. Although, hCGβ has been found to be a minor component in urine in most studies [95, 261], variations in urinary hCG results have been found when the levels of hCGβ are altered, as seen in cases of Down syndrome pregnancies and gestational trophoblastic diseases [208, 262]. While hCGβcf is extremely stable during storage, hCG dissociates into its free subunits faster in urine than in serum causing hCGβ levels to be elevated [123, 263-266]. Any change in the levels of hCGβ in the stored urine samples may affect measurements of total hCG immunoreactivity.

The range of HhCG results in serum samples of ≤ 24 weeks gestation was 66–194592 pmol/L (n=137) compared with urine concentrations of 28.5- 228,062 pmol/L (n=135) indicating a lower range of HhCG values in urine than in serum samples, though with very wide variations in concentration. The lower values and wider range in urine concentrations was also noticed for hCG, with urinary levels ranging from 2990-1213527 pmol/L and serum levels varying from 5118-761372 pmol/L. The levels of human chorionic gonadotrophin in urine reflect the renal excretion of circulating serum hormone either directly or after being metabolised in the kidneys. Variations in urinary flow rate lead to a wider reference range for hormone levels in urine compared to serum [95, 214]. The hCG concentrations in urine correlate strongly with those in serum. In early pregnancy, the average urine concentrations are 50–70%

of those in serum, but after the fifth week, the urine-to-serum ratio of HCG decreases and hCGßcf becomes the dominant form in urine [95]. There is large day-to-day and within-day variation in urinary hCG concentrations which can be reduced by normalizing against urine density or urine creatinine concentration. However, although this eliminates the variation, it partly limits its utility; therefore making serum assays better suited for quantitative assays [95]. Many previous reports confirm a similar trend of loss of hCG immunoreactivity in urine, accompanied by increased levels of degraded forms such as hCGß and hCGβcf [95, 113, 115 66, 68, 115].

Miscarriage is the commonest gynaecological emergency in England and Wales, comprising of 70000–90000 cases per year [267]. It is estimated that as many as 12%-15% of clinically recognized pregnancies, and as many as 17%- 22% of all pregnancies, result in spontaneous miscarriage [206, 268, 269]. The vast majority of miscarriage cases occur during the first trimester with evidence of the presence of genetic abnormality in the fetus in approximately 54%- 76% of cases [270-272]. Spontaneous miscarriage after 12 completed weeks of pregnancy (late miscarriage) is less common, but still affects 1–2% of pregnancies. Early and late miscarriages have been attributed to several factors including increased maternal age, ethnic origin, gravidity, parity, pro-coagulant state, luteal phase defect, and prior fetal losses [273-277]. However, approximately 40 % of pregnancy failures remain unexplained [184]. In the current study population, the incidence of miscarriage was higher with the increase of the maternal age and parity (Table 9).

Serum and urinary samples of 72 women from the UAE population whose pregnancy ended before 24 weeks of gestation (the "non-continuing group), showed lower levels of all hCG forms (Figures 28-36 and Table 14). These lower levels were observed irrespective of whether the patient was symptomatic or asymptomatic (i.e. presence or absence of the bleeding (Figures 42-43, Table 15) or the presence or absence of a dead embryo (Table 16), suggesting that the lower production of these hormones by the trophoblast is independent of embryonic development or the appearance of symptoms.

The cause of bleeding is unknown but it is said to occur in one fifth of recognized pregnancies before the 20th week, and over half of these miscarry, with the remaining half continuing despite the fact that bleeding may continue for a variable period of time [267]. In the UAE study population, in women with first trimester vaginal bleeding and a viable fetus on ultrasound (n= 28), lower concentrations of HhCG and related

hCG subunits were found in cases of negative outcome (i.e. almost a third eventually miscarried, n=10) than in those pregnancies continuing beyond 24 weeks of gestation. This is in agreement with previous observations [227,236,278] and with Harville et al who showed that the majority of pregnancies with bleeding progressed to full term (85%) despite the presence of bleeding often for a considerable period of time [279]. Women who presented with threatened bleeding during the first or second trimester, and who eventually miscarried (n=10) had lower HhCG levels in serum and urine than asymptomatic pregnant women (n=137). There was no difference in hCG levels between pregnancies with threatened bleeding but which eventually ended in live birth (n=13) and asymptomatic pregnant women (n=137). However, the results for HhCG in serum and urine as well as hCGβ in serum just failed to reach a 5% level of significance (p= 0.068, 0.086 and 0.089 respectively). Larger studies may show a significant difference between the two groups, which may indicate that bleeding is probably the earliest sign of placental dysfunction. As discussed previously [Section 1.12], survival of the implanting embryo depends on the extent of initial insult experienced during trophoblast invasion. Hence, the presence of additional oxidative stress-related damages leads to complications such as pre-eclampsia, FGR and fetal death. This, together with reports of the association of 1st trimester bleeding with impaired pregnancy outcome, is an independent risk factor for obstetrical and perinatal problems later in gestation [241,280,281], and may well confirm that bleeding is likely to be associated with some placental dysfunction.

In anembryonic missed abortions, serum HhCG, hCG and hCGβ levels were 3.3-, 1.8- and 1.2-times higher than in the embryonic missed miscarriage group. In urine, the HhCG, hCG and hCGβcf levels were 9-, 3-, and 2.4-times higher than in the embryonic missed miscarriage group (Table 16). This interesting finding shows that the production of HhCG by the cytotrophoblast, including the formation of other hCG-related molecules by the syncytiotrophoblast, continues regardless of fetal demise. Similar findings in the literature have lead to the concept that the presence of a fetus may not be necessary for the growth and development of a definitive placenta, since persistent placental function could be seen for some time, in spite of fetal demise during the second trimester [282]. Furthermore, complete hydatidiform moles with no fetus continue to secrete large amount of hCG. It may also highlight different pathophysiological mechanisms involved in pregnancy failure between the two missed miscarriage subtypes, with embryonic miscarriage probably caused by

placental dysfunction, reflected by lower levels of hCG forms, whereas pregnancy failure in anembryonic missed miscarriage may be precipitated by embryopathic factors (i.e. fetal-related causes). Indeed, trophoblast cell cultures obtained from pregnancies with a non-viable embryo (embryonic miscarriage) were severely impaired [282]. In contrast, anembryonic pregnancies were more likely to be aneuploid and fail at a very early gestation [271, 283].

Another possible explanation for higher hormonal levels in anembryonic miscarriage maybe due to stimulation of hormone production secondary to the hypo-perfusion caused by increased expression of oxidative stress markers after very early embryonic demise, exacerbated by the long delay before expulsion [184]. This has been confirmed by Doppler studies showing that only anembryonic miscarriage patients and those with long retention time, i.e. evidenced by the calcification of yolk sac, show abnormal intervillous space echoes [284]. As discussed earlier, the urinary hCG concentrations reflect the renal excretion of hormone directly from the circulation or after being metabolized from the liver. While all the hormonal levels of embryonic pregnancy in serum were higher than in urine (Figures 26 and 27), such trend was absent in anembryonic pregnancy (i.e. blighted ovum) indicating that hormones produced by blighted ovum are resistant to degradation and metabolic clearance from the circulation (Table 16). This may be due to an increase in the sialyl content, or due to the action of enzymes produced by macrophages in the trophoblast.

While several mechanisms are likely to be involved in normal and failed trophoblast invasion, a balancing act between the pro-invasive proteins produced by the trophoblast and local uterine factors acting to limit the invasion is mandatory for a successful implantation. As discussed earlier, hCG, cell adhesion molecules, matrix metalloproteinases (MMPs) and their tissue inhibitors, transforming growth factor β and its receptors, changes in the oxygen tension, and spiral artery transformation all play role in the initiation and maintenance of pregnancy. Similarly, HhCG may be a marker of inadequate placentation during the first trimester or may be, itself, a cause of pregnancy failure as a result of reduced invasive stimulation. This is due to the fact that high levels of HhCG are associated with aggressive trophoblastic invasive events during the early weeks following implantation [70] and in trophoblastic disease, [48, 49] making it a marker of invasive activity of the trophoblast. These and other studies have reported an association between reduced levels of HhCG during early pregnancy and adverse pregnancy outcomes, confirming that low levels of HhCG may be a

marker of inadequate placentation and other pathological events associated with trophoblastic dysfunction.

The six genes of the hCGβ gene family are expressed variably throughout pregnancy. Although the contribution of individual genes to the production of HhCG subunits is as yet unknown, it is thought that the total amount of hCGβ gene expression, rather than the expression of individual beta genes, is important for the maintenance of normal pregnancy [285]. A significant reduction of hCGβ gene expression is seen in cases of spontaneous miscarriages [285, 286]. Hence, it is possible that lower levels of HhCG and other forms of hCG molecules are due to a reduction in total hCGβ messenger RNA (mRNA). Henderson et al. found that the depressed concentrations of maternal serum hCG during early pregnancy failure resulted not only from diminished placentation and/or placental necrosis but also from down-regulation of hCGα and hCGβ genes [287].

In about two-thirds of early pregnancy failures, there is evidence of defective placentation mainly characterized by a thinner and fragmented trophoblast shell, reduced cytotrophoblast invasion of the endometrium and incomplete plugging of the lumen at the tips of the spiral arteries [288, 289]. Hence, this anatomical defect maybe reflected by the reduced levels of HhCG observed here in the "non- continuing pregnancy" group. Indeed, the impairment of the placental-decidual interface as a result of premature onset of the maternal circulation throughout placenta accompanied by major oxidative degeneration, causing further placental degeneration leading to loss of syncytiotrophoblast function and detachment of the placenta from the uterine wall [184, 289, 290] may be the cause of the lower levels of hCG, hCGβ and hCG βcf observed in maternal serum and urine samples in the "non-continuing pregnancy" population. It is difficult to establish in this study which of the above mentioned pathological events (i.e. impaired trophoblastic invasion or abnormal uteroplacental perfusion) preceded one another and which lead to miscarriage. The latter is more associated with late miscarriage rather than early miscarriage [271, 291]. However, in line with several previous reports, the results show that pregnancies which failed spontaneously before 24 weeks of gestation were all associated with lower levels of hCG molecular forms, including HhCG [67, 81, 242, 257, 292, 293]

There is presently no definitive test that can identify pregnancies that will spontaneously miscarry. In today's practice, low levels of hCG, non-doubling of hCG levels, or hCG levels that fall before the expected time of the hCG peak are used as

indicators of impending spontaneous miscarriage. Other markers including CA 125 [294, 295], progesterone [293, 296, 297], and PAPP-A [227, 298, 299] have been used, either as a single measurement or in combination with other markers, but have failed to discriminate pregnancy failures from ectopic pregnancy solely by means of hormone measurements.

Serial evaluation of serum hCG levels is one of the important strategies in the diagnosis and prognosis of early pregnancy. Monitoring the patient involves looking for increasing levels of hCG, and clinical decisions are facilitated by the well-defined curve of expected hCG rise first described by Kadar et al in 1981, showing the minimal rate of increase for a singleton intrauterine pregnancy (IUP) to be a rise of 66% in 2 days [300]. This study used a small sample however (n=20, CI 85%). Other investigators have redefined the lower limit of the normal hCG rise for a viable IUP to be 53% in 2 days (CI 99%), based on serial hCG curves obtained from larger populations of pregnant women presenting with pain with or without vaginal bleeding who eventually went on to have a viable IUP [220, 301, 302]. Studies of hCG doubling times indicate that serum hCG levels rise by at least 66 percent every 48 hours during the first 40 days of gestation in 85 percent of viable intrauterine pregnancies. Conversely, in 15 percent of viable intrauterine pregnancies the increase is less than 66 percent in a 48-hour period [220, 302]. The USA hCG Reference Service has reported cases with a less than 1.5-fold increase in hCG in 2 days that have nevertheless led to normal term deliveries. In cases of threatened miscarriage, the rise in maternal hCG is slower [220]. An increase of 50 percent or less in a 48-hour period is almost invariably associated with a nonviable pregnancy. In contrast, an inappropriately small increase in hCG merely indicates a nonviable pregnancy and does not indicate whether the pregnancy is intrauterine or extra-uterine. As the interassay variability of hCG is 10 to 15 percent, a smaller increase or a decrease may also possibly indicate that the levels have reached the plateau level [115, 208]. Due to these variations in doubling time, the search continues for a single hCG test or alternatively more sensitive marker, not only to distinguish normal pregnancies from failing pregnancies, but one that can be used as a better discriminator between ectopic pregnancy and spontaneous miscarriage.

3.7.1.2 Ectopic pregnancy

Ectopic pregnancy is the major cause of morbidity and mortality amongst women of reproductive age group, and the recorded prevalence worldwide has been increasing

gradually. Between 1976 and 1993, the incidence in northern Europe increased from 11.2 to 18.8 per 1,000 pregnancies and in Korea, 1 case per 20 to 26 pregnancies is seen, which is relatively higher than in Western countries [303]. In the USA, the incidence of ectopic pregnancy has increased from 0.5% 30 years ago to 2%, but the death rate has also fallen to 1 in 2000 detected cases of ectopic pregnancy. Admission to hospital for ectopic pregnancy increased from 17,800 in 1970 to 88,400 in 1989, reaching 1 case per 60 pregnancies in the United States [303]. In the UK, the incidence has slowly risen in the past decade from 8.6/1000 to 11 per 1000 estimated pregnancies but death rates have fallen slightly from 0.6 to 0.4 per 1000 cases of ectopic pregnancy [304]. The Confidential Enquiry into Maternal Deaths in 2004 found that in the UK maternal deaths from EP accounted for 3.4% of total deaths in 2001. Fifteen deaths directly attributed to early pregnancy complications were recorded from 2000-2002, of which 11 deaths (73.3%) resulted from ruptured ectopic pregnancy (7 tubal and 4 cornual). None of the cases were diagnosed before rupture [304]. Many studies have tried to explain these rises, linking them to improvements in diagnosis, the possible association with the rising incidence of pelvic inflammatory disease and chlamydial infections, the change in maternal age distribution, smoking, and IVF treatment [305-307]. An incidence of up to 6.5% is recorded amongst the IVF population [307]. Delayed diagnosis of EP can cause rupture of the Fallopian tube, and intra-abdominal haemorrhage, requiring blood transfusion and emergency laparotomy. Approximately 30% of women treated for ectopic pregnancy later have difficulty conceiving, and the rates of recurrent ectopic pregnancy are between 5%-20%. This risk increases to 32% amongst women who have had two consecutive ectopic pregnancies [305, 308-310].

Early diagnosis of unruptured ectopic pregnancy is paramount for preventing mortality, reducing morbidity, and preserving fertility. A quick and accurate diagnosis of ectopic pregnancy is desirable, but at times remains tricky, as no single noninvasive test is available that will definitely confirm the presence of an ectopic pregnancy, and since the diagnosis also involves the exclusion of an intrauterine pregnancy. Diagnosis and management of ectopic pregnancy includes laboratory assays such as serial quantitative hCG measurements, imaging techniques including transabdominal and transvaginal ultrasound, as well as surgical interventions (e.g. culdocentesis and laparoscopy) [305, 308, 311-320]. A simple pregnancy test is the first step

in investigating a suspected ectopic pregnancy combined with ultrasound parameters. Kadar et al were the first to recognize the importance of combining ultrasound with serum hCG measurements. They introduced the discriminatory serum hCG zone in 1981 [300]. This was defined as the minimal hCG concentration above which the sac of an IUP can always be identified by sonography. The diagnosis of an EP is made when an intrauterine gestational sac is absent and the serum hCG concentration is above the discriminatory zone. During earlier days when transabdominal ultrasound was performed, the discriminatory zone was set at between 6,000 U/L and 6,500 U/L but due to the improved resolution of sonography and the introduction transvaginal ultrasound, the discriminatory zone has decreased, bringing the optimal serum hCG cut-off level down to 1,500 U/L [314, 320]. Similarly, investigators have redefined the earlier lower limit of normal rise for a viable IUP from 66% in 2 days to be 53% in 2 days, based on serial hCG curve obtained later from larger population [220, 300]. However, the use of a more conservative cutoff for minimal rise in hCG, one as slow as 35% over 2 days, has also been suggested in literature [312].

A single measurement of serum hCG has shown to be not only potentially falsely reassuring but also unhelpful in excluding the presence of an ectopic pregnancy [292, 314, 317, 321-323]. In normal pregnancy, an intrauterine sac becomes visible on transvaginal ultrasound by 33 days of gestation, when serum hCG is 500-1000 U/L. A yolk sac is visible by day 38, followed by fetal heart motion by day 43 [320]. In other words, at 5.5 weeks of gestation, or with a hCG level of 2000 U/L (discriminatory zone), the sensitivity of ultrasound scanning to detect a normally developing intrauterine pregnancy reaches 100%. Therefore, when an intrauterine pregnancy is not identified by transvaginal ultrasound scanning and the hCG level is below the discriminatory zone, a nonviable pregnancy is diagnosed. Similarly, when serial hCG values do not rise or fall appropriately, an abnormal gestation is suspected. A review by Lozeau and Potter (2005) comparing the diagnostic efficacy of commonly used biochemical and ultrasound parameters for detecting ectopic pregnancy indicated that single levels of hCG or progesterone have low specificity and cannot differentiate between ectopic and intrauterine pregnancy, and serial hCGβ levels that do not increase appropriately are only 65% specific for the detection of ectopic pregnancy. Whereas combined transvaginal ultrasonography and serial quantitative hCG measurements are approximately 96% sensitive and 97% specific for diagnosing ectopic pregnancy,

therefore making the combined testing approach most optimal and cost-effective strategy for diagnosing ectopic pregnancies [308].

Unlike the pattern of hCG rise mentioned above for normal pregnancy, no single curve is available to identify patients with spontaneous miscarriage or ectopic pregnancy. Similarly, the expected rate of hCG or the normal decline for spontaneous abortions and ectopic pregnancy is also not well-characterized. The rate of decline is dependent on the initial hCG level, which has a mean clearance time of 12- 16 days [324]. Standard curves of expected hCG decline for gestations that were later confirmed to have resulted in complete spontaneous miscarriages showed a rapid rate of decline in 710 women with higher initial hCG concentrations ranging from 21% to 35% at 2 days, and a slower decline than this value was indicative of an EP wherein intervention was necessary to exclude this diagnosis [324]. The data presented in Table 20 was suggested by the authors as a rule of thumb to compare the drop in hCG over the course of time [324]. The average time for the serum hCG concentration to decline to less than 15 U/L is 33.6 days but may take up to 109 days depending on the initial hCG levels [308, 315, 325].

Initial hCG (U/L)	Week 1	Week 2	Days to hCG ≤ 5 U/L
250	93	100	12
500	94	100	13
1000	95	100	14
1500-3000	96	100	15
4000	97	100	15
5000	97	100	16

Table 20 Expected mean % decline in serum hCG levels indicating the higher the initial hCG value, the faster is the decline in levels. [324]

Another large study analyzing serial hCG levels in 200 women who were ultimately confirmed to have ectopic pregnancies showed that 60% of subjects had an initial rise in hCG, but 40% had an initial fall [311]. The rise in hCG for women with ectopic pregnancies (75% increase in 2 days) was slower than the mean increase reported for a viable intrauterine pregnancy. The decline in hCG for women with ectopic pregnancies (27% decline in 2 days) was slower than the mean reported for completed spontaneous abortion. However, 20.8% of women presented with a rise in hCG values similar to the minimal rise for women with a viable gestation, and 8% of women presented with a fall in hCG values similar to women with a completed spontaneous

abortion. They concluded that the hCG profile in 29% of women with ectopic pregnancy can mimic that of an intrauterine pregnancy or a completed spontaneous abortion and that a "normal" hCG rise (53% increase in 2 days) alone does not confirm the presence of intrauterine pregnancy. Although these curves aid clinicians in the prediction of failing pregnancies that may be managed expectantly, a draw back of these curves is the erroneous diagnosis or misclassification of spontaneously resolving EP-patients with patients of spontaneous miscarriages, both of which show a spontaneous decline of hCG to >5 U/L [312]. Furthermore, a slower rate of rise has been reported in women undergoing fertility treatment wherein care was advised while evaluating the change in hCG [248].

Two other entities in clinical obstetrics wherein interpretation of hCG values may become tricky are: 'heterotopic pregnancy' and pregnancy of unknown location (PUL). 'Heterotopic pregnancy' is one where an ectopic pregnancy coexists with an intrauterine pregnancy. The range of occurrence for this type of pregnancy is estimated between 1:7963 and 1:30000 in the general population, with the incidence rising to 1 in 100 in pregnancies conceived through IVF treatment [326]. This type of pregnancy is challenge to diagnose using hCG measurements since subnormal hormone production from ectopic gestation may be masked by the higher placental production from intrauterine pregnancy [318, 327]. The other phenomenon 'Pregnancy of unknown location' (PUL) is defined as having a positive pregnancy test with no sign of a pregnancy on transvaginal ultrasound (TVS). The availability of low-threshold hCG testing helps in the diagnosis of EP before the rupture, but leads to "inconclusive ultrasonographic scans" in which the intrauterine pregnancies (IUP) can be missed at initial stages that are too early to visualize or even in failing process. It is estimated that 11% to 43% of women who present to an early pregnancy unit are classified as having a "pregnancy of unknown location" (PUL) [323]. The varying prevalence of PUL might be attributable to the experience of the radiologist, quality of the ultrasound equipment, prior knowledge of the woman's risks and symptoms and the presence of physical factors such as uterine fibroids and multiple pregnancies and obesity [323]. Four clinical outcomes can be seen in PUL: a failing PUL, an intrauterine pregnancy (IUP), an ectopic pregnancy (EP) or a persisting PUL wherein there is a plateau in serum HCG levels without visualization of an IUP or EP [328]. It has been reported that biochemical indices (doubling serum hCG) can be used to predict which PUL become

IUP and failing PUL with PPV of 96.6 and 87.5%, respectively. However, these indices (i.e. a discriminatory zone >1500 U/L and/or sub-optimally rising serum hCG levels) are is not reliable for predicting PUL that become EP and persisting PUL [328].

Currently, clinicians apply different approaches not only to distinguish between viable and nonviable pregnancies but also to identify patients at high risk of having an extra-uterine pregnancy. The absence of a gestational sac on transabdominal sonography coupled with a serum level of hCG > 6500 U/L, or the absence of a sac on TVS. with hCG > 1500 U/L are considered indicators of ectopic pregnancy in 87% of cases, whereas a complex adnexal mass in the presence of a positive pregnancy test without a visible sac is highly suggestive of EP in 96.3% of cases [319]. Another study recommended a cutoff level of 2000 U/L in patients without an ectopic mass or fluid in the pouch of Douglas [321]. Serum levels of hCG correlate with the diameter of the adnexal mass because the increased trophoblastic placental bed and vascularity in the site of the ectopic gestation allow increased hCG secretion [308, 319]. A slight increase in the risk of tubal rupture was shown if the hCG concentration rises above 1300 U/L and the ectopic mass diameter increases to $\geq$ 24 mm [329]. However, another study looking at the relationship of serum hCG levels in unruptured vs. ruptured tubal ectopic pregnancies had shown that serum hCG by itself cannot predict whether a tubal ectopic mass is likely to rupture and there was no safe lower limit in hCG titer below which ruptured ectopic was not seen [322].

When considering all the above drawbacks, it is clear that an alternate biochemical marker is required for clinical care and medical diagnosis, the use of which will facilitate and optimize the care of women, particularly with an undiagnosed symptomatic first trimester pregnancy. In this study we tested the validity of HhCG measurements along with other hCG analytes (including their ratios), to see whether it can reliably detect EP. Levels of HhCG in this study in serum were significantly lower in 7 women with ectopic pregnancy (median 108 pmol/L; IQR, 62.7- 1027) than in those with a normal pregnancy (n=137, median=186850 pmol/L, IQ range: 70123-301922 pmol/L) (Figures 35 and 36). Similar finding was seen in urine samples of women with ectopic pregnancy (i.e. median=50.8 pmol/L, and interquartile range IQ of 31.8 -512 pmol/L) when compared with samples from normal pregnancy (i.e. median=7243 pmol/L, and interquartile range IQ of 1143- 23544 pmol/L).

Considerable variation in individual patient results was seen in urine in spite of corrections for the specific gravidity probably indicating that serum levels may be far better discriminators of EP than urinary concentrations due to the tighter distribution of values around the median. Serum and urinary levels of hCG showed similar statistically significant difference between ectopic pregnancy and pregnancies with uneventful outcome. Serum hCGβ was <250 pmol/L in all women presenting with ectopic pregnancy compared to >500 pmol/L in the vast majority of women with viable pregnancies. This is consistent with the findings of Holman et al. [330] and Borrelli et al. [292] All women with ectopic pregnancies in their study had hCGβ concentrations of less than 1250 pmol/L and 500 pmol/L, respectively. In the current study the median level of hCGβcf in patients with EP was 191 pmol/L, which is also far lower than urinary levels in uneventful pregnancy outcome patients. Borrelli et al. showed that a very high cutoff of 14201 pmol/L for hCGβcf gives 100% sensitivity, however this was at the expense of a very poor specificity of 48% [292]. Cole et al. indicated that no urinary analyte apart from hCGßcf was found to be a good predictor of ectopic pregnancy, and proposed that hCGßcf could predict up to 84% of all ectopic pregnancies with only 1% false positives [331]. The study group in this report is too small to show any statistical difference between the ability of HhCG and other hCG subunits to discriminate ectopic pregnancy (n= 7) from spontaneous miscarriages (n= 61). Moreover when performance of HhCG in serum was tested under the ROC curve (Figure 47), the AUC was 0.74 which according to model created by Duc et al. [217] is ranked as moderately accurate (±) diagnostic test (Table 18). Small number of ectopic pregnancy samples yielded wide confidence interval (0.5- 0.9) with a very high standards of error, making the findings of HhCG performance under the ROC curve less reliable. Since women with spontaneous miscarriages had dramatically lower concentrations of the hCG molecular forms in this study, similar to those seen in ectopic pregnancies, it is unlikely that these two entities would be distinguished from one another. A larger study is required to compare the abilities of HhCG and other hCG molecular forms to differentiate ectopic pregnancy from spontaneous miscarriage. HhCG, in combination with ultrasound and other serum markers may be a more useful test for ectopic pregnancy than current diagnostic algorithms.

Using a cut-off value of 13 μg/L, measurement of HhCG identified 73% of pregnancy failures (miscarriages as well as ectopics) at a 2.9% false positive rate [81], corresponding to a predictive value positive (PPV or prediction of failures) of 85%. The same serum cut-off value could predict 100% of EP with a similar PPV. When HhCG levels were compared with hCG results using the same false positive rate (15%), hCG detected 43% of failures with PPV of 39% concluding that a significant improvement in accuracy was obtained by measuring HhCG rather than hCG for the detection of pregnancy failures [81]. Applying the value of 13 μg/L (~316 pmol/L) in the population of current study gave a high sensitivity of 98% but at the expense of poor specificity of 57%. The performance was poorer in urine (i.e. 90% sensitivity and 40% specificity) (Figure 47 and Table 25) further confirming the non-reliability of urinary hormonal measurements for screening tests.

The observation of reduced HhCG levels in serum as well as urine of patients with ectopic pregnancy compared to normal intra-uterine pregnancy was most likely a result of reduced secretion of HhCG, due to derangement of trophoblastic division in patients with ectopic pregnancy [70, 100, 106, 222, 332]. Moreover, the sialic content of HhCG allows it to be more easily degraded by nicking or degradation enzymes in patients with ectopic pregnancy than in normal pregnant women. Likewise, HhCG may dissociate faster due to an increased activity of degradation enzymes in urine, and this may result in urinary levels being lower than the serum levels. It is unlikely that any of the previous two scenarios is the reason for lower HhCG levels in current study since hCGβcf, which is an end degradation product of hCG, did not contribute to the major immunoreactivity in urines of patients with EP when compared with normal pregnancies.

Not only is the diagnosis of ectopic pregnancy is dependant on hCG values, but its management is also dictated by serum hCG levels along with the clinical presentation and transvaginal ultrasound findings. Ectopic pregnancy can resolve spontaneously through regression or tubal abortion and expectant management is 47 and 82% effective. However, about 90% of women with ectopic pregnancy and serum hCG levels greater than 2000 U/L require operative intervention because of worsening symptoms or actual tubal rupture. There is also the risk of tubal rupture even if serum hCG levels are low and/or declining the concentrations fall below 15 U/L[308].

Expectant management is said to be more effective when the serum hCG level is declining and is less than 1000 U/L. Also other criteria such as an ectopic mass of less than 3 cm, no fetal heartbeat, and patient's compliance for the follow all play role in successful expectant management of ectopic pregnancy [308, 315]. Patients who do not meet these criteria are treated surgically, in most cases by laparoscopy. Surgical treatment is particularly appropriate for women who are haemodynamically unstable; however 8% of patients have been reported to have signs and symptoms of persistent ectopic pregnancy following the salpingostomy procedure (i.e. the gestational sac is removed without the tube) and follow-up hCG determinations are advocated until the levels in serum become undetectable [333, 334]. Medical treatment most commonly involves Methotrexate therapy (MTX), a folic acid antagonist that inhibits DNA synthesis in actively dividing cells, including trophoblasts. When administered to selected patients, it has a success rate of up to 94%. Again, the treatment success is dependant on the initial hCG concentration; the lower the hCG levels at the time of treatment initiation, the higher the success rate of methotrexate therapy. A meta-analysis of data for 1327 women with ectopic pregnancy treated with MTX confirmed that resolution was inversely associated with hCG level with treatment success rate of more than 90% when the initial level is below 5000 U/L. [325].

In summary, a balanced approach is needed to reduce morbidity and mortality from a nonviable gestation such as an ectopic pregnancy, alongside a more conservative clinical approach, allowing clinicians to distinguish between a viable and nonviable pregnancy with a very low likelihood of prematurely interrupting a desired pregnancy. Ultrasonography is the diagnostic test of choice, with limitations largely based on availability and the gestational age of the pregnancy. To date, no single-point cutoff value for hCG has been proposed as a discriminator of ectopic pregnancy. This pilot study suggests that a single measurement of HhCG (in serum and urine) may provide information on the fate of a pregnancy that is destined to fail, but no single-point cutoff could be used to pin point ectopics from spontaneous miscarriages. The study group in this report is too small to show any statistical difference, and future larger studies in combination with ultrasound or other biochemical markers are required using HhCG. Furthermore, serial quantitative measurements of HhCG may prove to be more useful test than a single measurement in detecting ectopic pregnancy and should be explored.

3.7.1.3 Molar pregnancy

Hydatidiform mole belongs to a spectrum of disorders known as gestational trophoblast disease. These are rare pre-malignant conditions that also include persistent invasive mole, gestational choriocarcinoma and placental-site trophoblastic tumor. Molar pregnancy is the most common form of GTD, with an incidence that varies from 0.5- 4.6 per 1000 pregnancies, depending on regional, ethnic, and socioeconomic factors as well as age and parity [335]. The trophoblastic lesions are characterised by a hydropic (ie. vacuolar) swelling of the chorionic villi, which is due to the poor development or regression of the villous vasculature that makes the drainage of fluid supplied by the trophoblast impossible, and by trophoblastic proliferation [335]. Based on gross morphology, histopathological features and karyotype, it is classified as either complete hydatidiform mole (CHM) or partial hydatidiform mole (PHM) (Tables 21 and 22).

Cytogenetic features	Complete mole	Partial mole	Choriocarcinoma
Karyotype	Paternal origin	Paternal/maternal origin	Aneuploidy
	46XX (90%)	69XXY (58%)	
	46XY (10%)	69XXX (40%)	
		69XYY (2%)	

Table 21 Cytogenetic characteristics of complete and partial moles and choriocarcinoma. [335]

Histopathology	Complete mole	Partial mole	Choriocarcinoma
Trophoblast cell type	CTB, STB & intermediate	CTB & STB	CTB, STB& intermediate
Trophoblastic hyperplasia	Diffuse	focal	Diffuse, necrotic and/or haemorrhagic
Chorionic villi oedema	Diffuse	focal	Not present

Table 22 Histopathological characteristics of complete and partial moles and choriocarcinoma [CTB, cytotrophoblasts; STB syncytiotrophoblasts]. [335]

CHM is characterised by diffuse hydropic swelling and trophoblastic hyperplasia of the syncytiotrophoblasts, cytotrophoblasts and intermediate trophoblasts on the chorionic villous surfaces. Most CHM are cytogenetically diploid, with a 46XX karyotype derived from the paternal genome [335]. The total absence of a maternal genetic contribution results in unopposed action of paternally expressed genes, leading to excessive trophoblastic proliferation and a very early cessation of embryonic development. Hence, no embryonal or fetal tissues can be found in CHM. In contrast, partial moles (PHM) are almost all ''diandric'' triploids, with 1 maternal set and 2 paternal sets of chromosomes; they present with two populations of chorionic villi, one with normal morphology, and the other with scattered hydropic villi and focal trophoblastic hyperplasia. The excess paternal genetic dose causes trophoblastic proliferation but is presumably kept in check by the maternal genetic contribution, permitting the fetus to develop much further than in complete moles, sometimes well into the second trimester [336, 337].

Histologic examination and secondary tests for ploidy or karyotype of the molar and nonmolar portions of the placenta provide definitive confirmation, though this may be subject to inter- and intra-observation variations [336]. Ultrasound can detect villous hydatidiform transformations, but provides no information on trophoblast activity [338]. Furthermore, during the second trimester, the ultrasound picture of placental molar transformation raises several diagnostic and management dilemmas in differentiating classical hydatidiform mole from complete mole in a multiple pregnancy, partial triploid mole, hydropic abortion, and focal benign villous hydatidiform (pseudomolar) transformation associated with Beckwith–Wiedemann syndrome [338].

15% to 28% of women diagnosed with complete hydatidiform molar pregnancy and 4-9% of women with partial moles are at risk of developing persistent gestational trophoblastic neoplasia (PTD) (i.e. trophoblastic activity remains after evacuation of the HM, as reflected by persistent low or even rising hCG concentrations in blood). These patients benefit from prophylactic chemotherapy, reducing the incidence of PTD to 4–12% [255]. As approximately 1 in 5 of these pre-malignant cases transforms into gestational trophoblastic neoplasia or choriocarcinoma [235, 339], correct diagnosis of HM and identifying women at risk for developing PTD following their evacuation, remains a challenge in a clinical setup. In the UAE cohort in the current study, there were 3 cases of molar pregnancy, of which two were complete and one was partial mole. Both diagnoses were confirmed by histopathology.

It is difficult to distinguish the hydropic abortion from hydatidiform mole (diagnostic tool) and to separate the low risk partial mole from the higher risk complete mole (predictive tool), using biochemical parameters. However, hCG measurements play an essential role in the assessment of prognosis following molar evacuation and for detecting recurrences of disease (monitoring tool). Multiple forms of hCG have been reported to exist in GTD including [207, 257, 340, 341] and several investigators have explored the utility of hCG analytes in the management of GTD. Jauniaux et al. found that the concentration of hCG and hCGβ was high (>2.5 MoM) in both complete and partial moles [338]. Berkowitz et al. showed that trophoblastic cells in complete and partial moles differed significantly in the manner in which they secreted the free subunits of hCG. Complete moles had significantly higher concentrations of hCGβ and an increased ratio of hCGβ to hCG than partial moles, whereas partial moles had higher levels of hCGα than complete moles along with an increased ratio to total hCG [342]. Both the free subunits and their ratios to total hCG were lower in normal pregnancy than in HM. However these findings were not helpful in distinguishing persistent disease from non-persistent disease [342]. They suggested that the ratio of hCGβ to total hCG represented the extent of differentiation and hyperplasia of the trophoblast. More promising results based on a larger study by van Trommel et al. showed that hCGβ in serum, and the ratios of hCGß/hCG + hCGß and hCGα/hCGß were excellent diagnostic tests to distinguish biochemically between hydatidiform mole and normal pregnancy at a 100% specificity level at more than 90% sensitivity. However, none of the investigated hCG parameters had adequate diagnostic accuracy in the prediction of PTD to justify the initiation of prophylactic chemotherapy treatment for its prevention [255].

The usefulness of hCGβcf in monitoring of patients with GTD was also explored since evidence was detected of its production by cancer cells in vitro, including choriocarcinoma cell lines and in the culture media of hydatidiform mole tissues, [343]. hCGβcf was shown to be a useful tumour marker in cases of metastatic placental site trophoblastic tumour, when serum hCG levels were near to or below the limit of detection [234]. In another study, serum hCGβcf levels rapidly declined and became undetectable after uterine evacuation in patients with hydatidiform mole with subsequent spontaneous resolution, but remained elevated or started to rise before persistent GTD could be diagnosed by a rise in hCG levels. While it was inferred that persistence of viable trophoblasts may be detected using hCGβcf, the same study had

shown that hCGβcf measurements may not be suitable for the follow-up of patients receiving chemotherapy, since it became undetectable more rapidly than hCG because of its rapid clearance from the circulation as compared with hCG [344].

The two cases of complete mole in this study had higher levels of HhCG, hCG and hCGβ in serum and urine, which is in agreement with results of Jauniaux et al [338] and Van Trommel et al [255]. The reason why higher hCG and hCGβ are synthesized in complete hydatidiform mole than in partial moles or normal pregnancy is unknown, but altered biosynthesis of hCG and irregularities in trophoblastic differentiation have been suggested [92, 338, 345, 346]. Jauniaux et al suggested that placental overgrowth could explain the increased maternal serum and urinary hCG concentrations whereas the opposite may be true for partial mole, where fetal overgrowth could overtake trophoblastic hyperplasia [338]. Furthermore, both trophoblastic hyperplasia and villous hydatidiform change are focal and milder in degree in partial moles [243]. This further supports why levels of hCG subunits may be lower in partial moles compared to complete hydatidiform moles.

Elliott et al. found higher levels of nicking of hCG in cases of persistent trophoblastic disease (trophoblastic neoplasia) and suggested that nicked hCG may be useful in the identification of trophoblastic neoplasia [18]. Data from a study by Kohorn et al. showed that at the time of presentation in 45 patients with gestational trophoblastic disease, more than 80% of hCG was intact and less than 20% was nicked. These proportions were reversed as hCG declined spontaneously after hydatidiform mole evacuation or following chemotherapy in patients with post-molar trophoblastic tumour or with metastatic trophoblastic disease. They concluded that the proportion of hCGn compared to hCG increased with the resolution trophoblastic disease. However they also inferred that measurement of hCGn did not seem to have additional clinical value in those few patients who had reappearance of their trophoblastic tumour as measured by an increase in total hCG immunoreactivity after an apparent disappearance of hCG to negative levels [119]. Nicking leads to rapid dissociation of hCG and HhCG, releasing hCGβn. In cases of trophoblastic disease and other hCG-producing tumors (testicular cancer and other germ cell malignancies), when hCG results fall below 100 U/L, the combination of nicked hCG and its dissociation product, hCGβn, become the major or sole sources of hCG immunoreactivity [347]. Considering the heterogeneity of hCG analyter in gestational trophoblastic diseases it is essential to use an hCG test that measures all forms of hCG and its free subunits as cases have been reported in which

a recurrence of invasive disease or persistence of a hydatidiform mole have been completely missed using an assay that does not detect nicked hCG or free β-subunits [341].

In most of the centres, hydatidiform moles are evacuated as soon as possible using suction curettage, and hCG is monitored on a weekly basis in order to diagnose metastatic sequelae as early as possible [348, 349]. A plateau or rise in hCG levels indicates persistent disease and the need for chemotherapeutic management. Complete remission is typically declared if the hCG level spontaneously declines to undetectable levels and remains there during a 6-month follow-up period [349]. According to FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) guidelines in 2000, the diagnosis of post-hydatidiform mole trophoblastic neoplasia should be considered when any of the following is observed [349]:

1. A plateau of hCG is observed over a period of 3 weeks or longer, i.e. for Days 1, 7, 14 and 21.
2. A rise in hCG level is observed for three consecutive measurements or longer, over a period of at least two weeks or more, i.e. on Days 1, 7 and 14
3. There is histological diagnosis of choriocarcinoma
4. hCG level remains elevated for 6 months or more

It is however important to exclude the possibility of heterophilic antibody interference [see Section 1.1.15.1] which may lead to inappropriate treatment if not recognized.

There are also guidelines for risk-factor scoring and a staging system that classifies untreated patients into distinct prognostic categories (high-risk or low-risk) so that treatment outcomes can be objectively compared. However, there is lack of uniformity in the use of these staging and scoring systems [211, 350]. In an effort to establish hCG levels that were predictive of outcome, several investigators have evaluated hCG regression curves post-evacuation to determine whether patients will go on to have persistent disease or go into remission [348]. However several centres recommended prognostic scoring systems, that were different from the others, are a result of differences in the study methods, treatment selection criteria, treatment regimens and outcome measures. Hence a universally accepted hCG regression curve like those available for ectopic pregnancy and miscarriage is unavailable [350, 351]. The decline in hCG levels after molar evacuation has an underlying biologic basis. Women with molar pregnancies who have trophoblastic tissue confined to the endometrial cavity are likely to have a rapid fall in their hCG level after the

trophoblastic tissue is removed at surgery and are at a low risk of developing persistent disease. In contrast, women whose trophoblastic tissue has invaded the uterine wall, or metastasized beyond the uterus, will have a slower fall in their hCG level after surgery due to the presence of the residual hCG-producing tissue and are probably at a higher risk of developing persistent disease. A slow decline in hCG levels after molar evacuation may, therefore, reflect the presence of invasive or metastatic trophoblastic tissue [352].

The new FIGO 2000 staging system combines anatomical staging and a modified WHO prognostic risk score, for stratifying patients into a low- or high-risk group and has suggested investigations for metastatic surveillance [349]. The system is a step forward in unifying the diagnostic and prognostic modalities between centres for the management of GTD, however the performance of this system in different clinical setups needs to be field-tested to see if such a goal has been achieved or not. Surveillance of the hCG levels remains the primary tool for clinicians to appropriately manage and treat GTD. While the prognostic risk score according to the new FIGO 2000 staging system stratifies low-risk group with the pretreatment level of hCG to be less than 1000 U/L (i.e. score 0) and high-risk group have pretreatment level of hCG to be less than 100000 U/L (i.e. score 4), yet there remains a grey area between low-risk and high-risk groups. Since HhCG is only produced by invasive cytotrophoblast cells during early pregnancy and by malignant forms of gestational and non-gestational neoplasms, its presence is considered to be a significant virtual marker of ongoing invasion and malignancy [70, 72, 74, 175].

Khanlian et al. evaluated 114 patients referred to the USA Reference Service with persistent low levels of hCG. Based on their findings, 45% of cases were found to have false-positive hCG results caused by interfering antibodies and the remaining 55% (63 patients), had real persistent low-level hCG titres (usually <50U/L) [200]. After prolonged surveillance of these low hCG titres, they found that the levels persisted despite chemotherapy or surgical interventions, and HhCG accounted for less than 21% of total hCG immunoreactivity amongst these women. However, in four cases that experienced abrupt and steep rises of persistent low serum hCG concentrations, HhCG accounted for 81%-100% of the detected hCG immunoreactivity and trophoblastic malignancy was confirmed pathologically. They further analyzed stored serum samples from women with confirmed gestational trophoblastic neoplasms and found that in all cases, HhCG immunoreactivity exceeded 30% of the total hCG

immunoreactivity. Their results lead to the conclusion that HhCG measurements may be used to differentiate preinvasive gestational trophoblastic disease with low hCG titres (quiescent GTD) from malignant disease [200]. Several subsequent reports from the USA Reference Service indicated similar results and showed that HhCG was a reliable marker to distinguish quiescent GTD from invasive disease [175, 235, 339]. However, the confidence of the USA Reference Service was not shared by two recent consecutive studies, by Duc et al [217] and Van Trommel et al [75]. In another study, a retrospective analysis of HhCG as a tumour marker for the prediction of PTD prior to the evacuation of molar pregnancy using receiver-operating characteristics (ROC) curve analysis showed that the diagnostic accuracy of HhCG was only moderate and no different from that of hCGβ and total hCG. Hence, it was inferred that HhCG and other hCG parameters tested had limited diagnostic accuracy for the prediction of subsequent development of persistent GTD [217]. In the second study, the time course and disappearance rate of HhCG following the evacuation of moles was compared longitudinally with total hCG (hCG +hCGβ) in serum samples from 3 groups comprising uneventful post molar regression, patients who developed GTD and were managed with mono-chemotherapy, and patients developed GTD and were managed with poly-chemotherapy [75]. They found significantly longer mean serum half-lives for total hCG and HhCG in the poly-chemotherapy group as compared to the mono-chemotherapy and the uneventful regression groups. However no significant differences were observed between the mono-chemotherapy and the uneventful regression groups. Since significantly shorter mean half-lives for HhCG than for total hCG were observed in all three groups of patients, they questioned the use of HhCG as a tumour marker due to its rapid clearance from the circulation, and suggested that it may not be suitable for following up patients after molar evacuation or who are receiving chemotherapy.

Clearly, identifying patients at increased risk for developing PTD after evacuation of hydatidiform mole is of great importance. It is challenging to find an adequate pre-evacuation diagnostic test that establishes a patient's risk of developing persistent GTN or of achieving remission within a few weeks of molar evacuation, or that can reduce the interval between the diagnosis of molar pregnancy and the diagnosis of persistent disease, and so allow initiation of chemotherapeutic management for specific high-risk groups whilst reassuring women who are at low risk of persistent disease. HhCG may be a promising candidate that can differentiate invasive from

noninvasive disease, since it is only produced by the invasive trophoblast cells [70, 244]. However, because of conflicting findings in the literature, the value of HhCG measurements in predicting PTD needs to be further established in larger prospective trials.

3.7.2 Hyperemesis gravidarum

3.7.2.1 Background to the condition of hyperemesis

Nausea and vomiting in pregnancy is a mild form of hyperemesis gravidarum, and is a common problem affecting 50% to 80% of all pregnancies within the first 4–12 weeks of gestation. It is characterized by episodic vomiting that is more pronounced in the morning (morning sickness). It is prevalent during the first trimester of pregnancy when both the placenta and the corpus luteum are actively secreting hormones and the body is adapting to the pregnant state, but also can be seen in approximately 9% of patients continuing beyond week 20 of gestation [353]. Between 0.3% and 2% of pregnant women develop hyperemesis gravidarum, which is characterised by intractable nausea and vomiting leading to fluid, electrolyte and acid–base imbalance, nutritional deficiency and weight loss often severe enough to require hospital admission hence compromising activities of daily living [169, 353].

Hyperemesis is associated with positive pregnancy outcomes such as a lower risk of spontaneous miscarriage, but has also been linked to adverse obstetric outcomes such as lower infant birth weight and fetal death [354, 355]. The data in the literature are conflicting. However, since excessive vomiting may cause nutritional deficiencies, maternal weight loss, fluid and electrolyte imbalances, concerns are raised about possible adverse perinatal outcome which has been the basis of extensive research.

Many different factors have been considered as a cause of hyperemesis gravidarum, including endocrine factors, hepatic dysfunction, changes in lipid metabolism, immunological factors, defective mesenteric nervous control affecting gastric emptying; nutritional deficiencies and psychological factors, but consensus the exact aetiology has not been reached [169, 356]. Due to the close temporal relationship between peak maternal hCG levels and peak occurrence of nausea and vomiting symptoms, hCG is often stated as the most likely cause of hyperemesis gravidarum [357-359]. Conditions such as multiple gestations, trisomy 18 and hydatidiform mole are characterized by higher production of hCG levels and are more commonly associated with hyperemesis gravidarum [360, 361].

Structurally, hCG is related to TSH, LH and FSH except that hCG contains a carboxy-terminal portion not shared by others [17, 90]. Because of its structural similarity to TSH, hCG is considered to stimulate the thyroid gland during pregnancy. Acidic molecular forms of hCG have more thyrotrophic activity than hCG. The degree of biochemical hyperthyroidism as measured by thyroid stimulating hormone suppression correlates closely with the severity of nausea and vomiting of pregnancy [362].

The rapid increase in HhCG concentrations in the early part of first trimester may be a factor in, or an early marker of, hyperemesis gravidarum. Therefore in this study, we investigated the association between hyperemesis gravidarum and HhCG levels (both in serum and urine), to see whether any such associations exist.

3.7.2.2 Methods used to assess hyperemesis in the UAE cohort

In a cohort of 137 patients recruited in the UAE with term delivery and no adverse obstetric or perinatal complication, 31 had hyperemesis (22.6%) (Study group). As all pregnant women admitted with hyperemesis gravidarum were at or below 14 weeks of gestation, they were compared with 66 healthy pregnant women without hyperemesis gravidarum of similar gestational age ($\leq$ 14) who also had term delivery and no adverse pregnancy effect (Control group). Pregnant women in the first trimester who could not take oral nourishment, had ketones in their urine and vomited at least four times a day were included in the study group. The inclusion criteria included persistent vomiting in the first 14 weeks of gestation, with ketonuria (2+ or more) (10 mg/ml) on dip stick examination requiring admission to the hospital. The data were analyzed for statistical significance using the student t-test (or the Mann-Whitney U-test when non-parametric data were involved) and significance was set at $P<0.05$.

The 6 hCG analytes were compared in two groups with respect to age, ethnicity, BMI, parity, gestational week on admission, and delivery outcome. Chi-squared tests have been used to determine if there is any association between these variables and the presence of hyperemesis gravidarum.

Patient characteristics	Non-emesis Group N= 66	Emesis Group N= 31	P Value (0.05)
Age • ≤35 • >35	60 4	29 2	P=1 (NS)
BMI • <25 • ≥25	24 42	18 13	P= 0.04 (S)
Parity • Primi • Multi (1-5) • Grand-multi (≥5)	23 33 10	14 15 2	P= 0.39 (NS)
Ethnicity • UAE • GULF • N.AFRICA • SE. ASIA • OTHER	29 18 7 11 1	14 12 1 4 0	P= 0.64 (NS)

Table 23 Demographic characteristics of the study group and their association with HG. S= statistically significant p<0.05, NS=non-significant. Note that the number of N in each group does not represent the total number of cases in the HG- and non-HG group, due to missing data. [HG, hyperemesis gravidarum].

3.7.2.3 Results for the UAE hyperemesis cohort

The average gestational age at admission for the hyperemesis group was 8.4 weeks and there was no significant difference in the characteristics of the two groups in relation to presence or absence of hyperemesis. However, the only evidence of association was seen for the BMI group; with the association of hyperemesis gravidarum in pregnant women with higher BMI (i.e. ≥25) (Table 23).

The level of HhCG in serum and in urine was higher in the hyperemesis group than in the non-emesis group, as was hCG in serum as well as urine and serum hCGβ. All measurements, apart from hCGβcf showed a statistically significant difference (Figure 50-53, Table 24).

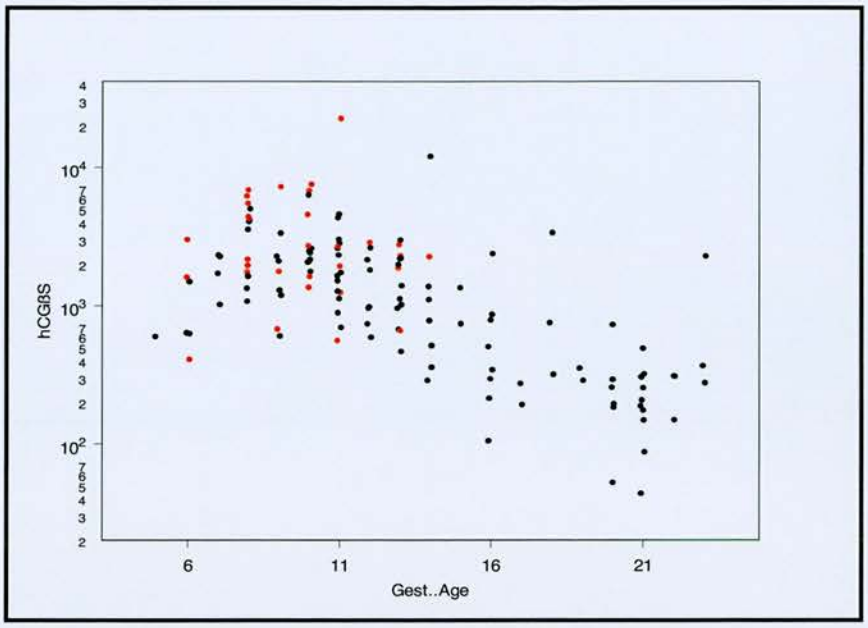

Figure 50 Serum HhCG levels of women with HG (●) vs. no HG (●).

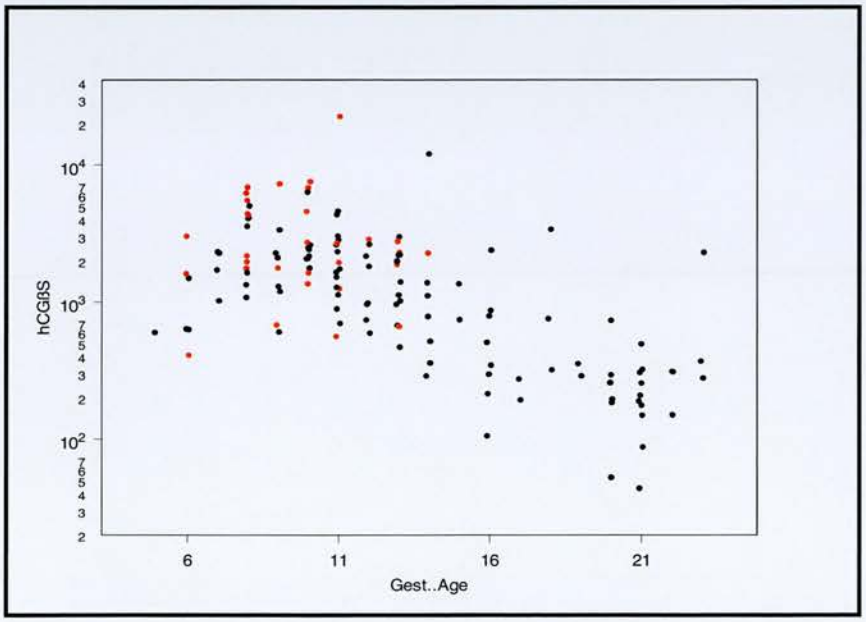

Figure 51 Urinary HhCG levels of women with HG (●) vs. no HG (●).

		HG = No		HG =Yes		95% CI for difference	P-value
		N	Median	N	Median		
Serum	hCG	66	204403	31	289633	(-152011, -37518)	0.003
	hCGβ	66	1671.3	31	2285.2	(-1536.6, -223.2)	0.008
	HhCG	66	24277	31	33673	(-22072, -1204)	0.032
Urine	hCG	66	90984	30	187154	(-146443, -29273)	0.002
	hCGβcf	66	206069	30	362669	(-248188, 23747)	0.165
	HhCG	66	10172	30	24479	(-18373, -2966)	0.006

Table 24 Levels of HhCG and other hCG molecular forms showing statistical significance.

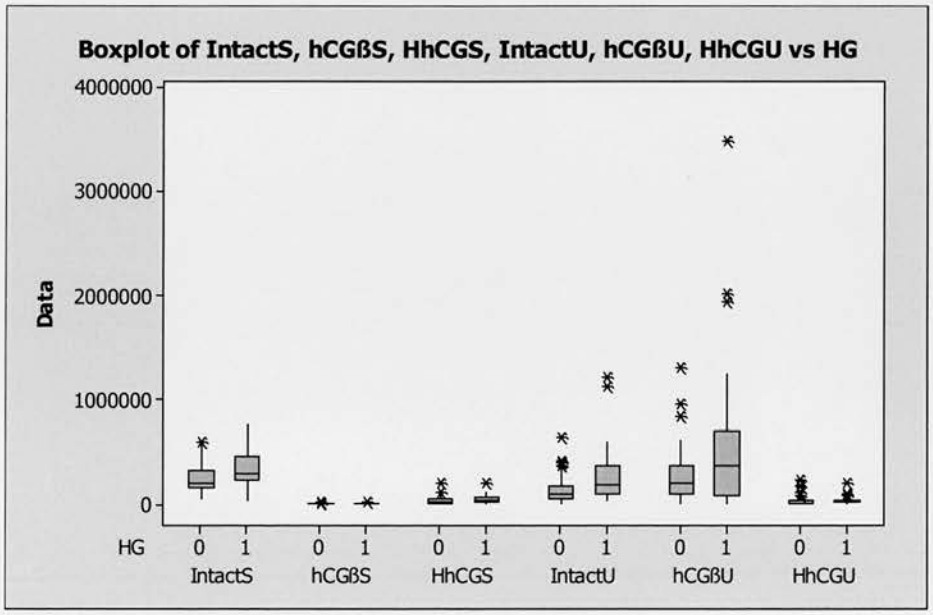

Figure 52 HhCG and other hCG subunit levels in serum and urine (0= Non-hyperemesis and 1= hyperemesis). Data includes maximum hormone levels. [IntactS, intact hCG (serum); hCGβS, hCG beta-subunit (serum); HhCGS, hyperglycosylated hCG (serum); IntactU, intact hCG (urine); hCGβU, hCG beta-subunit (urine); HhCGU, hyperglycosylated hCG (urine); HG, hyperemesis gravidarum.] [Median, inter-quartile range (box) and 10^{th} to 90^{th} centiles (whiskers) plotted. All asterisks indicate values outwith the whiskers.]

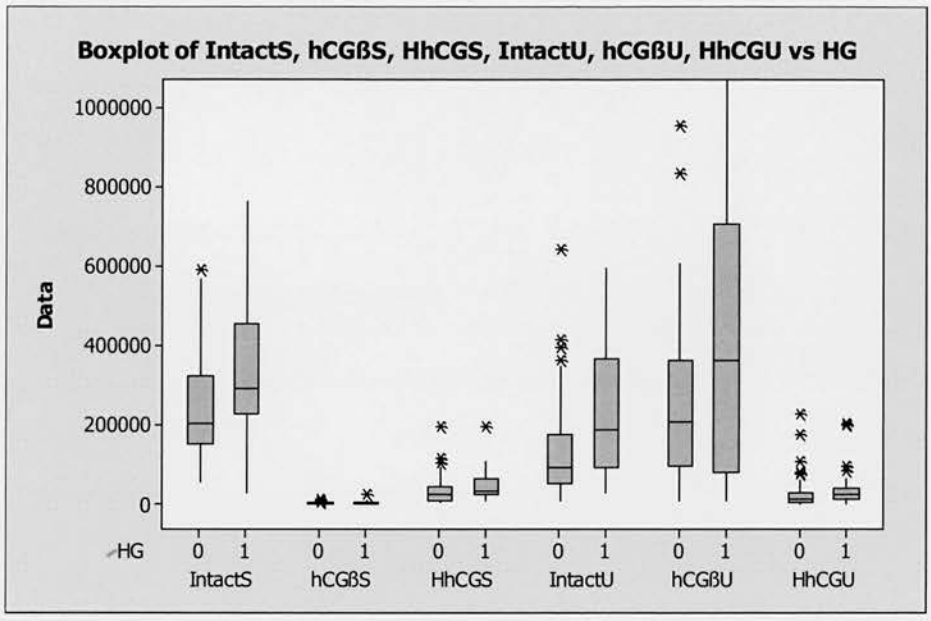

Figure 53 HhCG and other hCG subunit levels in Serum and urine (0= Non-emesis and 1= Emesis). [The maximum concentration for hCGβcf (coded hCGβu) in the graph was >10000000. The upper value therefore does not appear in the above graph.] [IntactS, intact hCG (serum); hCGβS, hCG beta-subunit (serum); HhCGS, hyperglycosylated hCG (serum); IntactU, intact hCG (urine); hCGβU, hCG beta-subunit (urine); HhCGU, hyperglycosylated hCG (urine); HG, hyperemesis gravidarum.] [Median, inter-quartile range (box) and 10^{th} to 90^{th} centiles (whiskers) plotted. All asterisks indicate values outwith the whiskers.] [Median, inter-quartile range (box) and 10^{th} to 90^{th} centiles (whiskers) plotted. All asterisks indicate values outwith the whiskers.]

3.7.2.4 Discussion of hyperemesis in the UAE cohort

Many endocrine factors have been hypothesized to be involved in the pathogenesis of hyperemesis gravidarum, including hormones of placental origin. It is not known if hCG directly or indirectly participates in the triggering of vomiting but several theories have been suggested.

Because of its structural similarity to TSH, increased hCG levels can cause excessive stimulation of the thyroid gland. It has been suggested that the high incidence of transient hyperthyroidism amongst patients with HG is caused by elevated circulating hCG levels either through triggering the thyroid hormone receptors hypersensitive for hCG or the production of a type of hCG that is more potent in stimulation of the thyroid gland [169, 358, 359]. During peak HCG levels in normal pregnancy, serum TSH levels fall and are a mirror image of the HCG peak. Free triiodothyronine (T3) and (T4) levels are significantly elevated at this time [169, 358]. Several reports show that free T3 or T4 levels are significantly elevated at the time when hCG levels are maximal. In a review investigating the relation between hCG and hyperemesis gravidarum that

compared circulating hCG levels in hyperemesis gravidarum patients, 23 studies were identified in a literature search between 1968–2004 [169]. 11 out of 15 studies published showed a significantly higher level of serum HCG in hyperemesis gravidarum patients than in controls. Patient who had hyperemesis gravidarum had higher βhCG concentrations and more heavily suppressed TSH. The highest suppression occurred during 9-13 weeks of gestation [359]. A prospective study of 57 consecutive patients with hyperemesis in Los Angeles compared hyperemesis patients with 57 women of similar gestational age [362]. Amongst hyperemesis patients, TSH was suppressed in 60%, free T4 was increased in 46%, while the free T3 index was increased in only 12%. Serum hCG levels were three-fold higher in the hyperemesis patients than in the controls. For the entire group, the degree of biochemical hyperthyroidism and the hCG concentration correlated with the severity of vomiting. An inverse correlation between the serum hCG and the serum TSH levels and a direct correlation of hCG with free T4 levels, has also been found [358, 362]. All of the above findings imply that hCG plays an important role in causing hyperthyroidism and is further supported by the finding of presence of thyroid hyperstimulation in cases of conditions with extremely high levels of hCG such as hydatidiform mole and multiple gestations [169, 360]. In a rare condition of hyperplacentosis, in which the placenta is enlarged and hCG concentration is very high, clinical hyperthyroidism has shown to remit promptly after delivery of the placenta [358]. In vast majority cases of hyperemesis gravidarum, the increased thyroid function spontaneously remits when the vomiting stops, usually during the later half of pregnancy [358], hence anti-thyroid drug therapy not required. Although some patients with hyperemesis, having frank clinical hyperthyroidism known as 'gestational thyrotoxicosis', may benefit from the treatment [363].

The rate of hyperemesis in pregnancy has been difficult to estimate. As many as 30% of all conceptions are thought to be lost before 24 weeks and 20% of these are not even clinically recognized, most databases exist at present as the ratio of hyperemesis to live births, rather than a ratio of hyperemesis to pregnancy rate. Also, many women have nausea and vomiting during their pregnancy that is considered normal and is treated as an outpatient. In Mafraq Hospital, all cases of hyperemesis gravidarum are first given anti-emetics as well as IV fluids in the A/E department. If electrolyte imbalance persists they are then admitted to the antenatal ward. Hence, only clinically meaningful hyperemesis, i.e. hyperemesis that was severe enough to require hospital admission, could be captured. In spite of not including out-patients, the rate of

hyperemesis in our hospital was 6.2% (62/1000 delivery). This is higher than previously published reports of hyperemesis gravidarum (0.3%- 2%) [169, 353] but it is in agreement with the findings in a Kuwait population, where an incidence of 4.5% has been reported. This is in accordance with reports that hyperemesis gravidarum is associated with low maternal age, primigravidity and high maternal weight [364]. Furthermore, the effect of long-term environmental factors or genetic influences has been explained for the differences in incidence of hyperemesis gravidarum between populations. A higher incidence of hyperemesis gravidarum has been observed in New Zealand Pacific Island women, United Kingdom Indian and Pakistani, Asian and African American women compared with ethnic European women, and lower incidences are found in American Indian and Eskimo populations [358].

Except for the hCGβcf, increase in the concentrations of all hCG forms in serum as well as urine in women with hyperemesis was found in this study (Figure 50-53 and Table 24). This may be secondary to excessive trophoblastic secretion by the expanding trophoblasts during the first trimester [70, 222, 252, 257]. As the levels of hCG in urine reflect renal excretion of circulating serum hormone into the urine either directly or after being metabolised in the kidneys [110, 344], it is possible that in hyperemesis gravidarum, the hCG produced is more resistant to degradation. Indeed, an alternate hypothesis for the appearance of transient hyperthyroidism during pregnancy suggests that hyperemesis gravidarum could be caused by a variant of hCG with increased thyroid stimulating activity rather than the absolute amount of hCG. This was supported by the finding that the hCG fraction containing asialo-carbohydrate chains was significantly increased in a population of hyperemesis gravidarum patients with gestational thyrotoxicosis compared with a control group of pregnant women with no emesis [365]. In addition, extensive variations in the sialic acid content of oligosaccharide side chains has shown to contribute not only to considerable heterogeneity of hCG, but also affects the biological activity of the hormone. It may be possible that hCG molecular forms produced in hyperemesis gravidarum have a higher sialic acid content which is known to protect the galactose residues of the carbohydrate chains from metabolic degradation by neuraminidases, resulting in less metabolic breakdown, hence, no difference in the concentrations of hCGβcf was found in this study between the hyperemesis and non-hyperemesis group [127, 366]. Indeed, hCG in serum from early pregnancy as well as from tumor patients, are often highly acidic [26, 47, 50] and studies indicate the acidic variants exhibit a dose-dependant

stimulation of T3 release and cAMP generation from human thyroid slices, hence act as functional stimulators of the human thyroid in vitro [367]. The evidence of significant increase in hCG fraction containing asialo-carbohydrate chains amongst hyperemesis gravidarum patients with gestational thyrotoxicosis compared with a control group of pregnant women with no emesis, further supports that presence of certain molecular forms of hCG, rather than an increase in the absolute hCG concentration, is responsible for the thyrotrophic activity, hence, nausea and vomiting during pregnancy [365]. Evidence of potent thyrotrophic activity of asialo-hCG in animal as well as human bioassays, has also been shown [358, 368]. While it is not possible in current study to ascertain this hypothesis since the differences in sialylation do not affect the overall reactivity of hCG in the immunoassay system or its detection by poly- and MAbs [47]. However, since HhCG, found in abundance in choriocarcinoma and during first trimester of pregnancy, also varies in its sialic acid content [18, 64], from the above presented arguments, it may be possible that HhCG may be responsible for hyperemesis gravidarum during pregnancy. Whether the action is through direct stimulation of thyroid axis or possibly via any other biologic effect, is yet unknown and should be a topic of further investigation.

3.7.3 Late pregnancy complications

Many pregnancy complications occurring late in gestation may have their origin very early in gestation, associated with abnormal placentation. A considerable proportion of pregnancy outcomes such as proteinuric and non-proteinuric pregnancy-induced hypertension (PIH), FGR, preterm labour, and stillbirths are associated with abnormal levels of hCG and other placental markers. There has been much debate in literature whether these are related or independent conditions, but most agree that they share some similar risk factors and are associated with high maternal and fetal-neonatal consequences [369]. Hypertension complicates approximately 9% of all pregnancies, with pre-eclampsia occurring in approximately 2% of pregnancies. World wide, 40 000 women die each year with hypertensive complications, with eclampsia contributing 2% to maternal mortality and 8% to fetal mortality [370]. According to the confidential enquiry into maternal deaths in the United Kingdom between 2000 and 2002 (Why Mothers Die, 2004), hypertensive disorders of pregnancy were second only to thromboembolism, accounting for 14 deaths (i.e. 8 maternal deaths from pre-

eclampsia and 6 from eclampsia)[304]. The incidence of FGR is increased 2- to 3-fold in women with hypertensive disorders, and the severity of hypertension correlates directly with the presence of FGR. Epidemiological studies even suggest that size at birth is related to the risk of developing disease in later life, particularly increased risk of coronary heart disease, diabetes, hypertension and stroke in adulthood [371-374]. Another important cause of neonatal mortality and morbidity with possible long term adverse effect later in life is preterm delivery. The reported incidence in the USA is 11%, 5%- 7% in Europe and approximately 7% in the UK [375, 376]. Estimates of preterm birth in developing countries have shown even higher rates (up to 25%), reflecting the geographic differences in the magnitude of problem [375-377]. Preterm birth is a major public health concern because prematurity accounts for approximately 56% cases of neonatal mortality and almost half of long-term birth-related neurologic morbidity [375].

An increase of 17% in the rate of preterm birth was recorded in the USA between 1980 and 2000 with similar increases in other countries such as Canada and UK [378]. Although much of the increase is attributed to the rise in multiple births as a consequence of assisted conception techniques, improved identification of at-risk pregnancies, closer monitoring of the fetus and earlier intervention may also have contributed to the increase. Coincidental with the increase in preterm birth has been a marked reduction in neonatal mortality: for England and Wales, neonatal mortality fell by 54% between 1975 and 1988 and by a further 20% between 1988 and 2000. As a consequence of improved survival, the proportion of children with cerebral palsy and other neurodevelopment problems who were born preterm has increased [378].

As discussed in chapter 1, many causes (i.e. genetic, nutritional, immunologic, and infectious) and their pathologic mechanisms (i.e. abnormal placentation, oxidative stress, and endothelial dysfunction) have been proposed, but due to the heterogeneous nature of the pathology of late pregnancy complications, prediction before the onset of signs and symptoms has been a challenge, with no single method being completely satisfactory. In a series of 5776 pregnancies, Muller et al. [379] showed maternal serum hCG (MoM) was higher in patients with small-for-gestational-age neonates and pre-eclampsia and his earlier report [380] showed, in addition to chromosome abnormalities, those with a hCG above the 95th centile were found to be at increased risk of

miscarriage, pre-eclampsia and premature rupture of the membranes. In one study serum hCG levels below the <10th percentile in the first trimester was associated with miscarriage, gestational hypertension, growth restriction, and gestational diabetes [299], and in another large study, a significant increase in PIH, preterm delivery, miscarriage and intrauterine fetal death but not in FGR, oligohydramnios or abruptio placenta was demonstrated [381]. Walton et al. [382] demonstrated that elevated hCG was associated with higher rates of stillbirth, PIH and preterm delivery, where as Basirat et al showed mean third trimester βhCG levels in pre-eclampsia were higher compared to normal controls [383]. Similar observations were seen using other hCG molecular forms such as α-subunit of hCG [191,222], urinary hCGβcf and nicked hCG [125,223,384].

Given the apparent association in the literature between abnormal hCG levels and adverse pregnancy outcome, we sought to evaluate whether first and second trimester levels of HhCG were also predictive of adverse outcome. If diseases occurring later in gestation originate from common patho-physiology of placental insufficiency and inadequate placentation, then the levels of placental hormone (including HhCG) may be altered. In group 1 of the UAE study population, consisting of 217 pregnancies continuing beyond the 24 weeks of gestation, 199 had a term delivery of which 137 were uneventful term delivery with no complication throughout pregnancy. The remaining 80 pregnancies were associated with adverse pregnancy complications including: proteinuric and non-proteinuric PIH, FGR, spontaneous preterm delivery, gestational diabetes, ante-partum haemorrhage, amniotic fluid index abnormality, intra-uterine death and neonatal death (Table 10), occurring either as isolated conditions or in combination with other pregnancy complications.

In contrast to the low levels of HhCG, HCG, and hCG in serum, as well as HhCG, hCG, and hCGβcf in urine seen in cases of spontaneous pregnancy ending before 24 weeks of gestation, levels in late pregnancy complications remained unchanged indicating their poor predictive value in detection of adverse pregnancy outcomes of later gestation (Figures 44 and 45). This finding is in agreement with many previous reports showing poor associations of urinary and serum hCG levels with adverse pregnancy complications. A study including 1,622 consecutive singleton pregnancies undergoing first trimester testing for Down syndrome showed lower levels of hCGβ associated with a higher incidence of spontaneous miscarriage, but this was a poor predictor of other pregnancy complications including, pre-eclampsia, preterm birth,

FGR etc [236]. Another larger study comparing 26,524 unaffected pregnancies with 3,728 pregnancies affected by low birth weight, FGR, preterm delivery and stillbirth, found that raised hCGß was not associated with an increased risk for any of the pregnancy complications investigated [231], and there are many other examples in the literature showing that the levels of hCG and hCG-related molecular forms do not predict the development of late pregnancy complications [224, 239, 240, 242, 385]. However, data regarding the usefulness of HhCG in the prediction of late pregnancy complications is scarce. As HhCG is secreted by invasive stem cytotrophoblast cells [70, 72, 244], most studies so far have focused on looking at its levels to determine early pregnancy outcome and identify pregnancy failures [65-67, 69, 81, 386], in the detection of Down syndrome pregnancies (which are known to be associated with poor trophoblast differentiation) [79, 80, 129, 201, 251, 387-389], and as a tumor marker for malignant or invasive gestational trophoblastic disease, to discriminate active from quiescent disease, and assess the need for chemotherapy [72, 74, 75, 175, 217, 260, 341, 390]. Only one previous study involving 568 spontaneous pregnancies undergoing mid-trimester genetic amniocentesis showed a significant correlation between low urinary HhCG levels and subsequent development of pre-eclampsia [71]. The current study failed to confirm this result as no statistically significant difference was found between the levels of HhCG and hCG-related molecular forms in uneventful pregnancy and pregnancy associated with hypertensive disorders. These data included 11 women who became hypertensive for the first time in the 20th week of pregnancy (pregnancy induced hypertension) and 9 women who had pre-existing essential hypertension. In the former group, 4 women (36%) developed pre-eclampsia (proteinuric PIH) and 33% of the women with pre-existing hypertension developed proteinuria (superimposed pre-eclampsia). For analysis, women with superimposed pre-eclampsia were considered together with the proteinuric- and non-proteinuric- PIH group, and essential hypertension was included with other medical disorders such as DM and thyroid disorders for analysis. This classification methodology may itself lead to a flawed result due to non-segregation of severe cases from the milder ones or early-onset disease from late ones, or by failing to take into account demographic variables known to be confounders of adverse pregnancy. Not only were the maternal and fetal outcome variables different within the syndrome of hypertension of pregnancy, but the underlying etiopathological mechanisms are thought to be different [391-393]. It is well recognized that first pregnancy, pre-pregnancy weight, previous history of

obstetric complications such as miscarriage, preterm and pre-eclampsia, chronic hypertension, and insulin-dependent diabetes mellitus, are all risk factors for the development of pre-eclampsia-eclampsia, all of which may be maternal triggers for the genesis of hypertensive disorders and which should therefore be separated from the placental genesis which is caused mainly by reduced placental perfusion [394].

Early onset of pre-eclampsia is associated with a higher risk of perinatal morbidity in subsequent pregnancies. Lopez-Llera and Horta examined 110 women in whom the onset of eclampsia was divided into gestational age ranges of less than 35 weeks, ≥35 to 38 weeks, and ≥38 weeks. With earlier onset during pregnancy, recurrent pre-eclampsia and small-for-gestational-age (SGA) infants in subsequent pregnancies were more common (42%, 15%, and 10% of infants were SGA in the three groups) [395]. Similarly, Sibai et al. showed that earlier-onset disease in first pregnancy was associated with more frequent perinatal death in subsequent pregnancies.

Perinatal mortality rates were likely to be four times higher extremely poor in the pre-eclampsia group compared to those groups with non-proteinuric gestational hypertension and chronic hypertension [396].

An immunopathological study on the placenta from women with hypertensive disorders showed no positive staining for α- and β-subunits of hCG in the syncytio-cytotrophoblast of placentas from mothers with spontaneous hypertension, and only weakly positive staining in mild pre-eclampsia. However, positive staining for hCG was seen in placentas obtained from severe pre-eclamptic cases [397]. Indeed, a recent immunostaining study of placentas from pre-eclamptic patients also confirmed a generalized increase in the secretory activity of the syncytiotrophoblasts [99]. Many clinical studies have confirmed that the severity of the disease is associated with altered placental hormonal levels. Lee et al. [398] demonstrated that midtrimester hCG levels were significantly correlated with the severity of pre-eclampsia, as women with mild pre-eclampsia had two and a half times greater chance, and women with severe pre-eclampsia had a six-fold greater chance of having hCG exceeding 2.0 MoM.

Underlying maternal diabetes and hypertension adversely affect perinatal outcomes, but pre-eclampsia superimposed on these maternal disorders has even greater adverse perinatal consequences. Caution is needed before pooling data into specific groups, as the presence of a mixture of disorders may lead to difficulties in analysis and produce heterogeneous and non-specific results. A systematic review by WHO evaluating pre-

eclamptic screening methods in use from 1996 to 2003 showed that the majority of screening tests (including hCG) have low predictive potential [399].

During normal implantation and the early stages of placental development, the cytotrophoblasts proliferate and invade rather than differentiate, under the influence of relatively hypoxic conditions, causing a rapid increase in placental mass than the embryonic lineages. Failure of endovascular invasion and inability of the cytotrophoblast to invade to the appropriate depth has been linked with spontaneous miscarriage, pre-eclampsia, and FGR[181-183]. Thus, from a biological stand point, obstetric complications that are associated with inadequate trophoblastic invasion during the first trimester may be associated with low placental hormone levels. The finding of low urinary levels of HhCG by Bahado-Singh et al. amongst the pre-eclamptic group, may be explained by the involvement of similar mechanism [71]. Conversely, the pathophysiological mechanisms discussed in chapter 1, existing alone or in combination with one another, may lead to elevations in the maternal circulatory placental hormone. The compensatory response due to the reduced blood supply, leading to reactive hyperplasia of cytotrophoblastic cells in an environment of low oxygen tension [181-183], along with aberrant morphological changes in the placenta leading to abnormal trophoblastic secretory response and hyperplasia of cytotrophoblast without the formation of syncytiotrophoblast [186-190] may all lead to an increase in the levels of hormones produced by the cytotrophoblast such as HhCG.

Another plausible mechanism by which levels of placental hormones may be increased is that abnormal/altered hormone metabolism and excretion, (either due to active degradation as a result of nicking and dissociation or due to conformational changes of the placental hormone) lead to rapid clearance. A significant increase in urinary hCGβcf levels in the pre-eclamptic group has been attributed to the activity of Elastase from leukocytes around the trophoblastic tissue which increase in pre-eclampsia and trophoblastic disease, leading to increased nicked levels of hCG which readily dissociates, releasing free α- and β-subunits and hCGβcf in urine [125, 257, 384, 400].

The oligosaccharide structure of glycoprotein hormones is important not only for folding, assembly and signal transduction, but also for heterodimer secretion and metabolic clearance [33]. The carbohydrate side chains, each ending with a sialic acid residue, stabilize the hormone, decreasing the dissociation rate and increasing the circulatory half-life [18, 123]. Differences in terminal sialylation between normal and aberrant pregnancies have been found [18] and the glycosylation pattern is affected by

the activity of different oligosaccharide-processing enzymes such as sialyl- and glycosyl-transferase, which have been shown to be altered in conditions associated with poor vascular supply such as pre-eclampsia [401], gestational diabetes [18], chromosomally abnormal pregnancies [401] and choriocarcinoma [35, 402]. In diabetic pregnancy, sialylation of both N- and O-linked oligosaccharides is significantly decreased compared to normal pregnancy [18], and in pre-eclampsia, the bioactivity of serum hCG was lower than in normal pregnancy, however, immunoreactivity tended to be higher [403]. When Frendo et al. studied hCG glycosylation in cultured trophoblast cells from second-trimester control- and trisomy 21 (T21) affected-placentas, they found abnormally glycosylated, highly acidic and weakly bioactive molecular forms of hCG were synthesized by T21 placental cells. Fucosyl-transferase-1 and sialyl-tranferase-1 transcripts were also abnormally high in T21 trophoblasts [401]. In another histopathological study, in choriocarcinoma cell lines formation of abnormal biantennary sugar chains of HhCG was shown to be due to high N-acetylglucosaminyltransferase-IV (GnT-IV) activity [35, 402].

The basic pathology of gestational diabetes remains unclear and debate continues over whether gestational diabetes represents a pregnancy-induced state of glucose intolerance or only the exacerbation of a pre-existing insulin resistance that would become evident in the future regardless of pregnancy [404]. There is no consensus on selective screening for GDM in different populations, nor is there universal agreement on diagnostic testing procedures. There is even uncertainty about the merits of its early detection and intervention, as the associated maternal and fetal consequences are less profound than in pregnancies with hypertensive disorders [405-407]. It is known that risk varies in different countries and in women belonging to certain ethnic groups, such as Asians and South Asians who exhibit a higher risk of developing GDM [2, 406]. In the current study from the UAE, 10.6% of the "continuing-pregnancy" group had pregestational diabetes and 6.4% developed GDM (controlled by diet or insulin) during pregnancy. This is in agreement with previous studies showing a high prevalence of diabetes and gestational diabetes (i.e. 20% and 11.3%, respectively) in association with maternal obesity and high-order multiparity in this significantly diverse multi-ethnic population [2, 408, 409]. In populations with a high prevalence of gestational diabetes (GDM), any form of oral glucose testing for screening or diagnosis excessively strains the health care system. Additionally, these tests are

conducted later in pregnancy because it is more likely that abnormal carbohydrate tolerance will be detected later in gestation since the physiological changes of pregnancy exert a greater strain on a woman's ability to maintain normal carbohydrate tolerance with advancing pregnancy. As a consequence of enhanced insulin production by the fetal pancreas following an excessive transplacental glucose load, infants of diabetic mothers may become macrosomic (defined as infants weighing more than 4,000 g at term), and are more susceptible to infant respiratory distress syndrome, cardiomyopathy, hypoglycaemia, hypocalcaemia, polycythaemia and birth trauma [410]. 8 out of 12 macrosomic infants seen in this study were born to mothers with diabetic disorders, amongst whom there was increased medical and/or operative delivery intervention, shoulder dystocia, spontaneous preterm and even one intra-uterine death. Effective screening is important for early identification of an individual's susceptibility to gestational diabetes before the onset of glucose intolerance, in order to prevent excessive fetal growth/ neonatal complications through early intervention. Therefore, the search continues for a biochemical screening test that can be performed sufficiently early in pregnancy to detect pregnancies at risk of GDM and discriminate low-risk from high-risk women, in order to avoid conducting a provocative glucose challenge for every pregnant woman in a population with high prevalence of GDM. Although uteroplacental metabolic derangements caused by deregulation of glucose and oxygen metabolites have been proposed to explain the altered levels of placental hormones and proteins that precede a diagnosis of GDM, none of them provide a sensitive prediction of gestational diabetes [404, 411]. This study too failed to demonstrate that HhCG levels (or any other hCG analytes), was altered in pregnancies with pre-existing diabetes or in those women who subsequently developed gestational diabetes. This is in agreement with many other previous reports [226, 382]. There is continuing controversy as to whether calculations should be corrected for AFP, unconjugated oestriol, and inhibin- A, most studies now agree that hCG and hCGβ are not significantly different in pregnancies with and without IDDM, indicating that metabolic derangements in diabetic pregnancy do not affect hCG levels [412-414]. Compared to hypertensive disorders, fewer studies have shown association between diabetic disorders in pregnancy and altered hCG levels.

A computerized search of the PUBMED database up to February 2007 excluding reviews, and using as key words "Hypertension", "pregnancy", and "hCG" vs. "Diabetes", "pregnancy", and "chorionic gonadotrophin") was published by Raty et al and demonstrated that women with GDM exhibit lower mid-trimester serum hCGβ and AFP MoM values than healthy controls [415]. Similar lower levels of βhCG, PAPP-A and inhibin A, during the first trimester were reported by Tul et al. [239], lower βhCG and PAPP-A by Ong et al. [299], and lower unconjugated oestriol and hCG levels by Palomaki et al. [416]. A comparative longitudinal study throughout pregnancy of maternal serum levels of hCGα, between well-controlled insulin-dependent diabetic patients matched with non-diabetic controls showed that α- hCG was significantly lower in pregnancies affected with IDDM compared to non-diabetic pregnancies until the 24 weeks of gestation, after which it was higher until delivery [417]. One possible explanation for the apparent difference in serum concentrations of hCGα is that low levels during the 1st and 2nd trimesters are a consequence of impaired placentation and smaller placental mass, whereas high levels in the 3rd trimester may be the result of hypoperfusion-related stimulation of production of this hormone reflecting a compensatory mechanism. The same study also showed the although hCGα levels did not correlate with fetal macrosomia, the duration of diabetes, PIH or premature delivery, increased levels of hCGα in type I diabetic pregnancies during the third trimester were found in pregnancies associated with pre-existing hypertension, suggesting that established diabetes may have a more profound effect on normal placental differentiation than diabetes appearing during pregnancy as it may share common pathophysiological mechanisms with those underlying hypertensive vasculopathy [417]. Indeed, placental vascular changes such as impaired relaxation, reduced nitric oxide synthesis leading to reduced blood flow and increased placental vascular resistance, and increased oxidative stress have been reported for both conditions [418]. Other morphological investigations of the placental bed in diabetic pregnancy have shown varying degrees of change in the syncytiotrophoblast, cytotrophoblast, trophoblastic basement membrane and fetal vessels [411]. There is evidence that diabetic pathophysiology is associated with an alteration of expression markers for terminal placental differentiation [419]. Moreover, ultrastructural and ultrahistochemical studies of placentas from women with pre-gestational diabetes have shown patchy focal syncytiotrophoblast necrosis with marked cytotrophoblast hyperplasia and focal thickening of the villous trophoblast basement membrane [411].

Placentas from well-controlled diabetic pregnancies are not heavier than those from normal pregnant women. However, diabetic placentas from the subgroup of poorly controlled diabetes particularly in the presence of macrosomia, have a larger fetal capillary bed of greater length, diameter and exchange surface area between mother and fetus. This is in contrast to the finding of incomplete/ or absent transformation of the decidual and myometrial portions of the spiral arteries found in diabetic women complicated by chronic hypertension and/or superimposed pre-eclampsia, and in diabetic pregnancies complicated by fetal growth retardation showing more frequent syncytial knots along with lower percentage of vasculo-syncytial membranes, and thicker trophoblastic basement membrane [411]. All of these data indicate that developmental changes of placenta secondary to pre-gestational or gestational diabetes are differentially affected depending on the active underlying pathophysiological mechanism, explaining why the discordant data in the literature on levels of hCG in diabetic pregnancies and why two different patterns of abnormal fetal growth (i.e. macrosomia and FGR) are seen amongst them.

FGR is known to originate from multifactorial heterogenous pathologies that make the task of its prediction extremely difficult. While the importance of normal placental function in regulating many aspects of embryonic and fetal development cannot be denied, the actual cause-and-effect relationship is too complex to recommend a single guideline for its detection [183, 190, 404, 420, 421].

Additionally, a growing number of studies show that up to 20% of FGR may be due to "fetal factors" including chromosomal abnormalities which may constitute up to 7% and fetal infections may be seen in up to 10% of FGR cases [420]. At the biochemical level, many studies have shown altered levels of placental hormones due to shallow or inadequate trophoblastic invasion, an underlying pathophysiological mechanism which is also attributed to other pregnancy disorders such as spontaneous miscarriage, pre-eclampsia and spontaneous preterm delivery. The majority of these studies associate higher [223, 238, 379, 422] or lower levels [423, 424] of hCG, hCGβ and hCG βcf with an increased risk of an adverse pregnancy outcome. As FGR is closely associated with pre-eclampsia, preterm labor and gestational diabetes mellitus, altered placental secretion of hCG may not give a clear-cut clue to the underlying pathology unless further tests are carried out. Current strategies to identify FGR foetuses involve performing early pregnancy scans for accurate dating, good antenatal surveillance in

the form of abdominal palpation and symphyseal-fundal height measurement followed by ultrasound biometry, with an emphasis on Doppler analysis as the most important tool to grade the severity of the fetal disease [421, 425]. Using ultrasound technology, FGR due to placental insufficiency can be diagnosed on the basis of reduced amniotic fluid volume, abnormal umbilical artery Doppler and evidence of growth failure on serial growth scans, provided that chromosomal abnormalities, malformations and infections are excluded. The role of biochemical screening remains controversial.

The cause of spontaneous preterm birth is multi-factorial, with infection being implicated in up to 40% of cases and the remainder are labelled as idiopathic [426]. In the UAE study population, 8% of women delivered before completed 37th gestational week, only 4% (n=10) of which were spontaneous. Current methods to identify patients at risk for preterm delivery have not been very effective with screening efficacies ranging from poor to moderate [427, 428]. These include plasma levels of oestradiol, progesterone, AFP, prolactin, corticotrophin-releasing hormone and C-reactive protein, and salivary oestriol [429, 430]. To date, the most effective marker for predicting preterm labor involves measuring cervico-vaginal fetal fibronectin levels, assessed serially or at specific gestational ages [431]. As hCG in both maternal serum and amniotic fluid is the result of hCG diffusion from the placenta throughout pregnancy, it has been suggested that the hCG level in vaginal fluid is a useful marker of preterm delivery [277, 427, 431-433]. hCG is present in high concentrations in amniotic fluid during pregnancy. The levels are similar to those in maternal serum in early pregnancy, then decline to 20% of maternal serum levels and follow a similar gestational pattern. From the time of conception, concentrations of hCG in amniotic fluid rise to peak (approx.54,000 U/L) between 8 and 12 weeks gestation and then decline to plateau levels (2,000 U/L) at approximately 18 weeks where they remain for the rest of the pregnancy [434]. Anai et al documented low and stable hCG levels, secreted by cervical glands, in the vaginal washings of normal pregnant women in the second and third trimesters [435]. The median hCG level in vaginal washings of women with confirmed premature membrane rupture increased 44- to 67-fold compared with normal women in the 2nd and 3rd trimesters. Their study concluded that, in the microscopic absence of blood contamination which might increase the levels of hCG in vaginal washings, measurement of hCG levels in vaginal fluid may confirm an accurate diagnosis of premature membrane rupture, in cases of equivocal membranes

rupture. Bernstein et al followed 77 women at high risk for preterm delivery through the course of their pregnancy, and compared the levels of hCG in the secretions of the cervix and vagina in those who delivered before 34 weeks gestation, and those who delivered at term [436]. A single hCG value >50 U/L obtained between 24 and 28 weeks' gestation predicts a 2-fold increase in the risk of delivery before 34 weeks gestation (P= .03). This cutoff value had sensitivity, specificity, PPV, and NPV for predicting delivery before 34 weeks gestation of 50%, 87%, 33%, and 93% respectively. This study confirms that the hCG level in cervicovaginal secretions may be a useful predictor (a negative predictor rather than positive one) of preterm delivery. A similar conclusion was drawn from a study by Guvenal et al., but the cut-off value of cervicovaginal hCG level used in this study (>28 U/L) had a sensitivity, specificity, PPV, and NPV of 87%, 65%, 28% and 97% respectively, for predicting preterm delivery [437]. A number of studies have shown increased rates of preterm delivery in relation to increased hCG levels [231, 381, 438-440]. However the reported sensitivities, specificities, positive and negative predictive values varied considerably and were far from optimal, some showing no association at all [236, 239, 299, 441].

The consequences of preterm delivery vary depending on prematurity, with a higher risk of neonatal mortality and morbidity for early preterm births (< 32 weeks of gestation) compared to those born between 32-37 weeks of gestation [375, 442]. As discussed earlier, the underlying mechanisms involved in the onset and severity of FGR and pre-eclampsia may also be involved in preterm birth bringing about variable outcomes depending on the timing and severity of the insult. Studying hormonal parameters in small numbers of spontaneous preterm births along with coexistence with other pregnancy complications in this study, not surprisingly lead to inconclusive results.

Whilst the involvement of hCG in the maintenance of myometrial quiescence is well established (reviewed in chapter 1), the role of HhCG in this regard is unknown. HCG exerts most of its biological functions by acting on the LH/hCG receptors found on luteal, endometrial, cervical and myometrial cells, and on many other reproductive and gestational tissues [160, 161, 443], whereas it is suggested that HhCG exerts a cytokine-like action involving the TGFβ receptors which are differentially expressed in the uterus and placenta [444]. TGFβ1 inhibits cytotrophoblast cell migration and invasiveness through the up-regulation of the tissue inhibitors of MMPs (TIMPs)-1 and -2, while TGFβ3 potently inhibits trophoblast outgrowth, and inhibition of TGFβ3

expression or activity results in increased outgrowth, elevated MMP production/activity and fibronectin deposition [444]. The pre-eclamptic placentas show some characteristics of reduced trophoblast invasion and increased expression of TGFβ3, the blockade of TGFβ3 restores invasive potential, a mechanism supporting the involvement of TGFβ3 in the pathogenesis of this disorder. Until future studies confirm this, similarity in the tertiary structure of HhCG with the transforming growth factor and other cytokines may indicate a likely role of HhCG in the maintenance of pregnancy and/or may be even involved the pathophysiological mechanisms of pregnancy disorders such as pre-eclampsia, FGR, and preterm labor. This needs to be the subject of future investigations.

The use of hCG and its related molecules, (or any other placental marker for that matter), in the prediction or the diagnosis of any of the late pregnancy complications remains unproven, because of their low predictive value, coupled with discordance in study results caused partly by the heterogeneity of the underlying pathology. In the wake of such diversity, and the small number of such late pregnancy complications in the current study cohort, it is not surprising that no significant association was found between levels of HhCG and the incidence of proteinuric and non-proteinuric pregnancy induced hypertension, gestational diabetes, FGR, preterm births, oligohydramnios, polyhydramnios and stillbirths ($p < 0.05$). It is unlikely that a single marker or hCG alone will be useful. The combination of serum markers and uterine Doppler ultrasound improves the identification of women at risk for subsequent pregnancy complications [422]. Although studies have shown that the sensitivity of screening improves when hCG isoform measurements are combined with other biochemical tests such as inhibin A, unconjugated oestriol and uterine artery Doppler, there is disagreement regarding the accuracy of these tests and their predictive values. A study by Wald and Morris gave a 55% detection rate with a false-positive rate of 5% using a combination of inhibin-A, hCGβ and unconjugated oestriol during the second trimester of pregnancy [445]. Another study estimated a pre-eclampsia detection rate of 49% with a 10% false-positive rate if free hCG hCG was used in combination with inhibin-A before 20 weeks [446]. Prediction of pre-eclampsia in a study by Spencer et al. using uterine artery Doppler ultrasonography and maternal serum pregnancy-associated plasma protein-A, hCGβ, activin A and inhibin A at 22 + 0 to 24 + 6 weeks gestation had detection rates of 50%, 5%, 10%, 44%, and 35% respectively, with a

false-positive rate of 5%. However, when these markers were used collectively, the test detected 75% of patients who subsequently developed pre-eclampsia, with a similar false-positive rate [447]. Large systematic reviews and meta-analysis studies have shown that, in spite of the many studies showing an association between abnormal serum markers and late adverse pregnancy outcome, the sensitivity of these tests is too low or modest to be applied for generalized screening [448-450].

It may be concluded that, due to the heterogeneous nature of the pathology of pregnancy complications caused by various etiological factors proposed in the literature, and lack in the exact mechanisms underlying such pathologies, coupled with unpredictable placental response in the wake of any complication and the consequent production of heterogeneous hormones, all together make screening for pregnancy complications extremely difficult before the onset of signs and symptoms with no single method being completely satisfactory.

3.7.4 Screening performance of HhCG in spontaneous conception

The significance of HhCG level predicting pregnancies which may eventually miscarry was compared with other common forms of hCG in urine and serum. HhCG measurements showed only moderate diagnostic accuracy for the prediction of pregnancies ending before 24 weeks of gestation compared to normal pregnancies and when compared to the remaining 5 hCG analytes, all of which showed good diagnostic accuracy. With AUC values in the range of 0.698-0.831 (0.764 ±.034) in serum and 0.675-0.814 in urine (0.744 ±.036), HhCG was a worse predictor of adverse pregnancy outcome than hCG and hCGβcf in both serum and urine.

Recently, Sutton-Riley et al. studied the utility of serum HhCG measurements in the diagnosis of early pregnancy failures [81]. They found that using a serum HhCG cut-off value of 13 μg/L they could identify 73% of all pregnancy failures at a 2.9% false-positive rate. Using the same cut-off in urine they reported 75% detection at a 15% false positive rate. When we tested this level in our study population after converting it to the equivalent unitage [213] (HhCG 1 μg/L = 24.3 pmol; 13 μg/L = 316 pmol/L), HhCG detected only 30% of all failures below 24 weeks of gestation but had an excellent sensitivity of 98% (2% false-positive rate). In contrast to the study of Sutton-Riley et al., a cut-off value of 316 pmol/L, had poor diagnostic efficacy in this study population, although the positive predictive value was high (Table 25).

	Sutton-Riley et al.	Current Study	Sutton-Riley et al.	Current Study
	Serum	Serum	Urine	Urine
Pregnancy outcomes	**Term outcome (n=87)**	**Pregnancies ≥24 weeks (n=217)**	**Term outcome (n=139)**	**Pregnancies ≥24 weeks (n=211)**
Corresponding cut-off concentration	13 µg/L	316 pmol/L	13 µg/L	316 pmol/L
Corresponding detection rate of failures (specificity)	n = 33	n = 71	n = 28	n = 69
a. All failures	73%	30%	75%	41%
b. Spontaneous abortions only	71% (n= 29)	42% (n=61)	(70%) (n= 20)	(45%) (n=59)
c. Ectopic pregnancy only	100% (n= 4)	57% (n= 7)	88% (n= 8)	40% (n= 7)
Sensitivity (all continuing)	85%	98%	52%	90%
Area under ROC curve ± SE	0.88 ± 0.003	0.764 ± 0.034	0.83 ± 0.049	0.744 ± 0.036
ROC 95% confidence interval	0.83–0.99	0.78–0.90	0.73–0.92	0.67–0.81

Table 25 Comparison of results using an HhCG cut-off concentration of 13 µg/L (~ 316 pmol/L) for predicting pregnancy failures in the current study.

Sutton-Riley et al. used the Nichols Advantage automated assay for the detection of HhCG so the observed differences in results may reflect differences in the assay procedure or may be due to other analytically related factors as discussed below.

A highly sensitive biomarker accurately identifies viable pregnancies (true positives) and a highly specific biomarker identifies nonviable pregnancies (true negatives). Clinically, it is critical to minimize false positive test results that incorrectly suggest the absence of non-viable pregnancy (miscarriages and ectopics) and falsely reassure the clinicians and patients. However this is achieved at the expense of decreased sensitivity and more false negative results. In other words, the cost of unnecessary intervention and close monitoring of unnecessary intervention with continuing pregnancies but are falsely identified as a miscarriage or non-continuing pregnancy is weighed against the health risks and hazards of falsely identifying a non-continuing pregnancy as a continuing one. When the optimal cut-off value was chosen to maximize specificity to detect 70% of all pregnancy failures, a serum HhCG level of 4846 pmol/L (~ 200 µg/L) had a sensitivity of 66% (i.e. 66% detection of the "continuing pregnancy", or the predictive value positive). In urine, the same cut-off

value of 200 μg/L yielded a better detection of failures with a specificity of 78% but with a lower sensitivity of 55%. Using the same cut-off value in serum, 100% of ectopic pregnancies could be detected but at the expense of a very low sensitivity (33%), showing high NPV to correctly identify miscarriages.

In summary, our study of a UAE population showed that HhCG levels were decreased in the miscarriage group. However the diagnostic accuracy of the given measurements is at best qualified as moderately applicable in predicting adverse pregnancy outcome. Other forms of hCG, particularly serum hCG itself, were more reliable markers.

3.7.5 Limitations of the UAE cohort study

3.7.5.1 Factors related to the study cohort

This study has a number of limitations. As the UAE samples were collected from spontaneous pregnancies, there were insufficient data to evaluate HhCG levels before the 6th week of gestation. HhCG is produced by the cytotrophoblast, and accounts for the majority of the hCG immunoreactivity in serum and urine (up to 100%), at the time of trophoblast invasion during implantation and weeks following it, with their proportions rapidly declining with advancing gestation as a result of trophoblast differentiation. This time interval is crucial to understanding the pathophysiological mechanisms underlying the origins of many adverse pregnancy outcomes resulting from defective implantation and placentation, which remains mostly uncaptured in most studies of spontaneous conceptions. Trophoblast invasion is known to occur in two phases, one which is very early during the pregnancy and a second wave occuring during mid-gestation. Hence any pregnancy complication arising from a failure of the first wave of invasion may exhibit altered hormonal levels for a short time only, after which the cellular factors controlling the expression of hCG molecular forms may be overtaken by compensatory mechanisms as gestation advances. Also, when evaluating hormone values at 6- 24 weeks of gestation, as in this study, changes in their levels occurring as result of a growing pregnancy may not be differentiated from altered levels in the presence of disease. There is an exponential increase in placental hCG concentration during the 1st trimester[115] and throughout the first part of the second trimester, and concentrations of hCG then decrease to approximately one-fifth of the maximum by the 16th week of gestation and remain at this steady level until term [95, 115]. Relying on a single hCG cut-off value for the prediction of adverse

pregnancy outcome using combined first and second trimester samples would be ineffective and decrease the confidence interval of a result. For the same reason, biochemical screening is normally performed during the early part of second trimester, when the variation in hCG concentration is minimal.

This is a prospective study and therefore some data and variables that may be of importance are incomplete. As discussed earlier, different pathophysiological mechanisms may be operative in the onset of some pregnancy complications which may differentially affect placental function, the pathophysiology of extreme premature delivery may be different from moderately premature delivery [239, 440, 441]. The same may apply for low birth weight infants [239, 451]. There was insufficient information about the severity of disease or gestational age at the onset of each complication, nor any information about structural or chromosomal abnormality in the fetus, placental weight and other variables to identify pathophysiological mechanisms of underlying disease and explain changes in the levels of HhCG and other related molecules in relation to that [226].

Many co-variables are known to influence first- and second-trimester markers, including maternal body weight, gravidity, parity and fetal gender, ethnicity, insulin-dependent diabetes mellitus, together with previous pregnancy screening results and other pregnancy morbidities. Marker alterations due to these variables may lower the detection rate of pregnancy complications [2, 404, 440, 452-454 412]. However, data regarding the effects of such variables on HhCG levels is scarce. In this study no statistically significant difference was found between pregnancy outcome and other variables, including previous miscarriage, ethnicity, BMI, blood group, haemoglobin and fetal sex, except for age and parity, where 45% of women in the "non-continuing" pregnancy group were more than 35 years of age compared to 21% of women less than or equal to 35 years of age in the continuing group. The risk of spontaneous miscarriage increased as parity increased (almost double amongst grand multiparas). Since it is unknown whether these two variables, or any other variable, could influence HhCG concentration. Thus, the only adjustment done in this study was to correct for specific gravity of the urine, due to the lack of information on baseline values and normal population-based parameters which may affect HhCG levels in the UAE population. Only one previous population-based study in the same geographical region was conducted to establish distribution parameters of first-trimester screening markers in Saudi and non-Saudi women [455]. Their results showed that the βhCG and

PAPP-A values decreased with increasing maternal body weight and significantly differ in various ethnicity backgrounds, with MoM values for hCGβ 25.2% higher in Africans and 19.4% higher in Orientals but 6.8% lower in other Arabian and Asian (by 5.8) women [455]. Such ethnic variations and the need for correction were also reported previously [453, 456-458]. For a proper interpretation of data and to minimize the variations that may arise from confounding variables, risk estimates and data analysis should be made after correcting for known variables.

The UAE community is composed of diverse individuals from varying cultural, religious, and socioeconomic backgrounds. According to the 1995 census (reviewed by Malik et al. [409], the total population of the UAE was 2.7 million, of whom 20% were UAE citizens, 25% were from other Arab countries, 50% were of South Asian origin (predominantly from India and Pakistan) and 5% were from other countries, mostly European and East Asian. The marked heterogeneity among this population makes it a high-risk group for adverse obstetric and perinatal outcome (Multi-ethnic high risk population) [2, 409].

In populations that were deemed at high risk for pre-eclampsia, the use of biochemical markers has a low predictive accuracy [448, 459]. A study of the Danish population assessing the risk of adverse obstetric outcomes in pregnancies with elevated maternal serum AFP or hCG levels according to pre-pregnancy risk assessment showed no differences between women classified as high- or low-risk of developing obstetric complications [454]. However, any differences have been attributed to differences in the population groups being studied, or alternatively, differences in defining what constitutes high risk [439].

As current obstetric practice calls for screening in low-risk populations, there is much interest in investigating cost-effect, sensitive screening markers of low-risk populations. A combined algorithm of urinary HhCG with ultrasound biometry was shown to be substantially superior to the traditional triple screen and to the quadruple screen tests for the detection of Down syndrome in high-risk populations [202], and showed equally high screening performance in a small group of women who were below 35 years of age. However, it was suggested that larger prospective multi-centre trials should be conducted to test its screening performance in a low-risk population [202].

Research based on cohorts identified by neonatal birth weights or birth centiles should be interpreted with great caution as misclassification of newborn infants could occur

for FGR. FGR is diagnosed when birth weight is below the 10th percentile of that anticipated for a particular gestational age. In most cases, FGR is confirmed by one or more of the following findings on U/S: low amniotic fluid index and fetal movement, pathological uterine umbilical artery Doppler pulsatility index, or decreased growth velocity on serial ultrasonogarphic biometry (at least a 14 day interval between measurements) [460]. The dilemma arises when early interruption of pregnancy occurs due to spontaneous preterm labour or to CS due to abnormal antenatal fetal heart traces. In such cases the provisional diagnosis cannot be confirmed ultrasonographically, hence the clinician must rely only on birthweight centile. Placental findings (including excess villous trophoblast, thickened trophoblast basement membrane and villitis following delivery) can confirm FGR; unfortunately, histo-pathological reports of placental examination were not obtained in this study. The main problem with this definition is that it will include many healthy small-for-gestational age (SGA) fetuses that are constitutionally small, and hence, it lacks the sensitivity to identify small infants arising as a result of abnormal intrauterine growth or placental dysfunction [420,461]. Up to 70% of SGA infants are constitutionally small simply due to maternal ethnicity, parity, weight, or height [420]. Hence, it is probably more appropriate to use lower centile limits, (e.g. less than the 3rd percentile, or more than 2 SD below the mean weight for age and sex) [461] or ponderal index, (defined as birth weight in grams divided by the cube of the height in centimeters) [420] as a better indicator of FGR in newborn infants. It has generally been assumed that fetuses experiencing a sub-optimal environment such as nutrient limitation or placental dysfunction during the first and early second trimester tend to be proportionally small in both weight and length at birth (known as symmetric growth restriction). In contrast, growth restriction that begins later in pregnancy results in disproportionate asymmetric growth restriction (i.e. preservation of head growth occurs at the expense of total body weight gain) [420,461]. This has been disputed in the literature, but evidence of different etiopathogenic mechanisms operating at different developmental stage of fetal growth and their impact in the development of symmetry/asymmetry FGR, exist. Patients with either early onset severe pre-eclampsia or pre-eclampsia superimposed on chronic hypertension prior to 30 weeks gestation produced more symmetric than asymmetric FGR infants [420] and in the Dutch winter famine of 1944-45, a severe nutritional insult in the last trimester resulted in a low ponderal index at birth [372]. Hence abnormal placental marker levels such as HhCG or other hCG subunits may

depend on the stage of development and the type of FGR. Furthermore, the risk of low birth weight was increased when the difference between the observed and expected crown–rump length was -7 to -2 days. Outside this range, the risk of low birth weight was similar to the average for the whole group. They explained that these differences are most likely due to an incorrect estimate of post-conception age which largely obscures the variation related to growth [462]. Unlike pregnancies conceived through in vitro fertilization, the exact post-conception age at the time of ultrasonography was unknown in our study. The size of the embryo or fetus in the first trimester may also differ from the expected size because of variation in the timing of ovulation. If ovulation occurred on day 17 and was followed by normal conception, implantation, and growth, the fetus would be the equivalent of 3 days smaller than would be expected if ovulation had taken place on day 14. If this fetus was then born 41 weeks after the last menstrual period, gestational age would be the post-conception equivalent of 40 weeks and 4 days, but the infant's birth weight would be judged by the 41- week percentile [462].

Estimation of gestational age in spontaneous conceptions is not straight forward. In cases of miscarriage, there is a tendency to underestimate age of gestation if embryonic or gestation sac measurements are used as 30–70% of early miscarriages are smaller than expected based on the date of the LMP [284,463]. This could be related to several factors, including retention of conception even after fetal demise, growth disorganization or slower growth rate of an abnormal embryo in miscarriage [283,464]. However, dating of gestational age using LMP has its own drawbacks, as it is difficult to determine the exact timing of ovulation, especially amongst women with irregular bleeding and those who are lactating [465-467]. Even among women who have regular menstruations and are certain of their LMP dates, variations in day of ovulation still occur and the use of LMP is likely to overestimate the true gestational age [465,468,469]. Last menstrual period estimates of the duration of gestation are subject to both random error and a systematic tendency to overstate the duration of gestation, most likely because of delayed ovulation [470]. All of these may not only affect the diagnosis of FGR but also influence the diagnosis of preterm birth. The limited number of FGR and spontaneous preterm delivery cases in the current study, may have contributed to the inconclusive results.

Whether preterm delivery was due to maternal or fetal cause (i.e. congenital malformations, structural defects, or secondary to pre-eclampsia etc) was not clear. In

one neonatal audit from the UAE, the mortality rate among very low birth weight babies was higher and in 54 neonatal deaths out of 8083 live births (0.67%) weighing ≥ 500 grams, problems of preterm births, lethal malformations and asphyxia accounted for 95% of the deaths, and half the malformations were autosomal recessive syndromes [471]. Another annual report from the UAE cohort showed that 40% of infant mortality was caused by congenital anomalies, and 35% of cases were associated with maternal disease during pregnancy [472]. These observed high fetal mortality rates were attributed to a high proportion of women of advancing age, along with grand-multiparity, a high proportion of traditional consanguineous marriages, and the lack of termination of pregnancies affected by fetal defects, due to religious constrains. All these factors reflect the vast array of pregnancy complications inherent to the heterogeneous UAE population.

Nine still births and neonatal deaths occurred in our study. This was not representative of the general UAE population prenatal mortality rate (9.49 per 1000 live births) [472]. Again the higher rate was due to collection of samples from patients who referred to the tertiary Fetal Medicine Unit of Mafraq Hospital and were already receiving care while being admitted to an antenatal ward for conditions known to be associated with adverse pregnancy outcome such as diabetes or hypertension. 8 of the 9 fetal deaths could be explained by an apparent direct cause or were associated with a maternal condition known to have a high maternal and fetal mortality and morbidity, such as hydrops fetalis (n=3), placental abruption associated with pre-eclampsia, placenta previa, uncontrolled maternal diabetes. Only one fetal death was unexplained, hence the association between HhCG levels and stillbirth is too weak to reach any statistical significant conclusion.

3.7.5.2 Factors related to analytical procedures

The effect of storage and transport on the stability of these analytes over the time must be considered in assessing the results achieved in this study, given that the serum and urine specimens were stored for up to 3 years at -80°C until assayed.

Besides the heterogeneity of hCG and its related molecules and variations in the assay methods, the differences in the assay results have also been attributed to the integrity of these molecules in blood and urine samples during transport and storage, especially in urine in which hCG molecules have significantly greater stability problems than in serum. Early studies of the stability of HhCG show that it is stable at room

temperature for up to 3 days, and can be stored at 2-8 C for up to one week from the sample collection [202]. When pure hCG (Batch P8 from pregnancy urine) and pure HhCG (Batch C3-II from choriocarcinoma patient urine) were added to non-pregnancy urine samples and stored for up to four weeks in room temperature, they dissociated slowly at a rate of 1.6% and 4% per week, respectively [201]. Long term stability studies have shown variable results. No clear difference was found in HhCG values when tested in nine fresh as well as frozen urine samples and no change was found in HhCG MoM values or in Down syndrome detection rates in urine samples when stored for up to three years in the freezer [473]. They suggested that storing the urine as well as serum samples at -70°C does not affect the HhCG immunoreactivity over the period of 3 years. HhCG was stable in pooled urine and serum for a minimum of 10 months at -70°C. Furthermore, HhCG was stable for 3 days at room temperature and 7 days in the refrigerator, as well as after two rapid freeze-thaw cycles [76]. However losses in HhCG concentrations were seen after multiple freeze-thaw cycles when the analyte was present in high concentrations [202]. This was evident in the study of Down syndrome specimens, wherein fresh urine samples were tested before and after freeze-thawing and showed approximately 50% reduction in the immunoreactivity when compared with specimens from pregnancies of normal karyotype [202]. These losses were more evident when the analyte was present at higher concentrations [202]. It was suggested that the reduction in the HhCG level in stored Down syndrome urine specimens was probably due to differences in the carbohydrate structure and conformation changes that make HhCG more susceptible to loss when urine changes state during freezing and thawing.

Higher concentrations of HhCG in this study during the first trimester than the second trimester (Figure 34-35, Table 15) as well as higher proportions of HhCG in urine than in serum (Table 13) agrees with the overall data available data in literature on HhCG [66, 67, 69, 130]. Furthermore, the serum and urine samples in the current study were stored in -80 C and were shipped on dry ice to their final destination for the analysis, it is unlikely that any variation in the temperature, that may have lead to slow freeze-thawing, had taken place to have caused relative changes between analytes due to the degradation, disassociation or other changes attributable to storage. Degradation of hCG in urine and serurm samples in vitro leads to loss in the immunoreactivity via nicking and/ or dissociation. These two possible mechanisms occur more rapidly in urine due to the action of proteases present in urine samples and the addition of

antimicrobials, diminishes nicking and slows the hCG dissociation pathway[122, 126, 263]. Adding sodium azide (5%) in all urine samples in the current study, may have helped in the stabilization of hCG and its related molecules by impeding the possible action of proteolytic enzymes and preventing microbial growth or contamination during variation in the temperature, if any.

Another possible explanation for variation in HhCG results could be due to a change in the composition of hCG carbohydrate side chain as a result of variations in the sialic acid terminal residues which can be found in some adverse pregnancy complications. This is seen in cases of Down syndrome in which the sialic acid content is altered, causing loss of recognition of HhCG in some assays [29, 474]. Although variations in the number of HhCG-sialic acid residues would affect the lectin-based assay more than the conventional immunoassays, which are relatively unresponsive to carbohydrate changes [49, 77, 475], yet poor detection of sialic acid deficient-HhCG has been reported in Nichols Advantage automated HhCG assay system [474].

Glycosylation profile of hCG indicates a wider range of glycoforms, with evidence of the presence of hyperglycosylation on hCGβcf and α-subunit of hCG [18, 82, 115]. Since the B152 antibody used in this study recognizes the core 2 glycan of hCGβ [63, 64], presence of hyperglycosylation elsewhere in any of the pregnancy complication may also result in a change in response of the assay. HhCG itself may exist in a variety of forms, similar to the hCG forms found in neoplastic conditions, such as nicked HhCG or nicked hyperglycosylated free β-subunit [390]. Hence, any variations in results may probably be due to differences in the affinity/avidity of binding to different nicked forms of HhCG to the B152 antibody. Cole et al. had shown that majority of commercially available hCG assays, whether for serum testing in a laboratory or for point-of-care use either under- or over-detected HhCG leading to a potential source of hCG test disparity [68, 69, 208, 390]. Furthermore, differences in the Down syndrome detection rates between assays were also attributed to the differences in their incubation time, with microtitre plate assay, which has a longer incubation time, yielding much better detection rate than the Nichols Advantage automated assay with shorter incubation [80, 389, 474].

Pure HhCG (choriocarcinoma hCG batch C5), known to be 100% nicked, is calibrated by aminoassay analysis and was used to standardize the assays in many studies [18, 65, 69, 208], where as in this study, JEG3 choriocarcinoma cell line HhCG standard was

used which is not nicked [49, 130, 180]. The variability in results may be attributable to differences in calibration. Results for various forms of hCG may be compared by expression of concentrations in mol/L and the equimolar recognition of the major hCG molecular forms is suggested to be essential in improving between-method comparability for hCG. The immunoreactivities of the molecular forms based on molar concentrations can be estimated on the basis of their molecular weights and the ratio between content in mass units and IU of the 3rd International Standard (IS) (i.e. 1 μg of hCG = 9.28 IU = 26.7 pmol) [84, 213, 476]. To this end, conversion factor for HhCG has not yet been established. When analyzed by the above method, Stenman in an Editorial paper calculated the mean value of HhCG as; 900 U/L = 97 μg/L = 2590 pmol/L (i.e. 1 μg of HhCG = 26.7 pmol/L) [213]. The HhCG conversion used in current study (1 µg/L of HhCG = 24.3 pmol/L, based on the MW=41000 Daltons) is fairly close to the value calculated by Stenman. Since the given molecular weight (MW) of HhCG in literature varies from 39,000- 43,000 Daltons, the discrepancy in values tend to occur depending on the MW used in conversion formula. Results reported by Cole et al. suggest that conversion factors differ among different methods [208].

In this study, significant differences in concentration of HhCG were observed between two immunoasay methods (DELFIA and Quest). The availability of a formal HhCG standard would assist in improving between-method comparability. However, using common pure hCG preparation help to minimize between-method variation but does not eliminate it [208, 260, 477, 478].

The potential for error can be noted with extremely concentrated or dilute spot urine samples. In the majority of urine studies [129, 251], HhCG levels were normalized for urine concentration wherein HhCG concentration (mg/L) was divided by the spot creatinine concentration (mg/g creatinine). A relationship was observed between normalized hyperglycosylated values and creatinine concentration. Samples with low creatinine concentration gave unduly high creatinine-normalized values, and those with high creatinine concentration were giving unduly low creatinine-normalized values (i.e. up to 7.3- fold difference in HhCG values) [129, 251]. An equation was derived to correct the error using following formula: c' = (0.877c) + 0.107, where (c) is the actual creatinine concentration and (c') is the corrected value. Alfthan et al. evaluated paired urine spot samples and serum samples from 21 women and found that urinary concentrations of hCG corrected by specific gravity matched serum hCG concentrations [215]. In the current study urinary hormonal concentrations were also

normalized using specific gravity (SG). However, since urine specimens collected from the UAE population were spot samples rather than first morning voids, and exhibited a wide range of densities due to hydration status and time since last micturation, it is possible that SG normalization may have led to overestimation or underestimation of values in samples with high or low specific gravidity. This could explain the elevated serum and urinary levels of all hCG analytes in women with Hyperemesis in this study, except for the hCGβcf levels which remained unchanged. Smaller elevations were more evident molecules with tighter than with wider distribution of values [479]. Due to the wide variation of hCGβcf level in urine (4463-3490085 pmol/L), when compared with HhCG (28.5- 228062 pmol/L) and hCG (2990-1213527 pmol/L), any alteration in their levels may not be readily evident in an immunoassay. Furthermore a slight increase in level may be masked by SG correction. Currently, the majority of screening studies use spot creatinine levels to normalize urinary hormonal levels, which is thought to be superior to SG for correcting values of HhCG and other hCG related molecules. Further studies comparing both methods are required to establish which is superior. Also, some studies have reported diurnal variations in the secretion of hCG hormone and its metabolites along with the influence of time of sample collection on the levels of hormone [32, 113, 480, 481]. Future studies should explore the true effect of time of day and correction of extremely dilute or concentrated samples on their final algorithms.

CHAPTER 4. HYPERGLYCOSYLATED HCG LEVELS FOLLOWING EMBRYO TRANSFER (THE EDINBURGH COHORT)

4.1 Background to the Clinical Study in Edinburgh

The early phase of embryonic development is critical for successful implantation and pregnancy survival and involves balanced interactions between fetal and maternal hormones, cytokines, growth factors, cell adhesion molecules and transcription factors [144, 183, 482]. Various studies have addressed the question of early hCG values and their potential use in the prediction of pregnancy in the IVF population [483-487]. However, serial evaluations of hCG along with U/S confirmation have so far been the mainstay as the standard tool for following up the viability of pregnancy achieved through IVF. Pregnancies obtained after in-vitro fertilization or intracytoplasmic sperm injection IVF/ICSI and embryo transfer (ET) are at risk of adverse outcome compared with natural conceptions, and hence require careful follow-up [488-490]. The common management of patients with suboptimal hCG levels involves several follow-up visits for repeated hCG tests which is not only time consuming and costly, but also places extra psychological pressure on an already vulnerable group [491]. Thus, an early marker that can accurately distinguish viable from non-viable pregnancies before the verification of a live intrauterine pregnancy by transvaginal sonography, and minimize the need of repeated follow-up samplings, is highly desirable amongst the IVF population.

HhCG is produced by the invasive cytotrophoblast cells, very early during pregnancy and in-vivo and in–vitro studies have shown that HhCG promotes cytotrophoblast and choriocarcinoma cell growth and invasion [72, 74]. Since invasion is vital for successful implantation, the levels of HhCG may indicate successful implantation or identify very early pregnancy abnormalities.

A retrospective observational study of an IVF cohort was undertaken to explore the relationship between HhCG levels on day 14 following the oocyte retrieval (OR) and pregnancy outcome, and to evaluate its usefulness (along with other hCG molecular forms) in the prediction of adverse pregnancy outcome in this IVF population. The levels of hCG and free- beta-subunits were also examined in order to compare the diagnostic ability of these hCG-related molecules in the detection of adverse pregnancy complications amongst the IVF population.

4.2 Study Protocol

4.2.1 Ethics

Ethical approval was obtained from the Local Research Ethics Committee and written informed consent was obtained from all participants prior to sample collection and storage (See Appendix).

4.2.2 Study group

Serum collected in the year 2005 from 160 women undergoing IVF/ICSI treatment in the Assisted Conception Unit (ACU) in the Edinburgh Fertility Centre, Scotland, were used in this study. Only women with a known pregnancy outcome after the completion of an IVF/ICSI–Embryo Transfer (ET) cycle were included.

Serum samples were analyzed for HhCG, hCG and hCGβ from which outcomes were available for 128 women and considered for this study. The recorded causes of infertility were: male factors (42%), tubal factors (14%), endometriosis (6.25%), unexplained (37.5%) and mixed male and female factors (11.7%). The median (range) age of the participants was 35 years (Table 30).

4.2.3 Treatment protocols

Patients in the ACU underwent the IVF/ICSI and embryo transfer according to protocols described in [218, 491]. Pituitary down regulation with a gonadotrophin-releasing hormone agonist (intranasal buserelin GnRHa) (Suprefact, Shire, Hants, United Kingdom) was followed by ovarian stimulation using human menopausal gonadotrophin (HMG) or follicle stimulating hormone (FSH) (Puregon, Organon, UK, or Gonal-F, Serono, UK, respectively). Transvaginal ultrasound (TVS.) and serum oestradiol (E_2) concentrations were used to monitor the IVF cycle. When three or more follicles reached a diameter exceeding 17 mm, 10000 units (500- 10000 IU) of HCG ((Pregnyl, Organon, UK)) was administered subcutaneously to simulate the LH surge of a natural cycle and induce ovulation. Oocyte aspiration was performed approximately 35–36 h after the hCG injection using a standard transvaginal ultrasound guided approach. Oocytes are fertilised in vitro by conventional IVF or by single intracytoplasmic sperm injection (ICSI). The embryo then was transferred on day second (D2) or third (D3) after the oocyte aspiration and a maximum of two embryos were transferred. All patients receive had luteal phase support with

progesterone for 4 weeks, either as vaginal pessary or rectal suppositories (200 mg BD) (Cyclogest, Shire Pharmaceuticals, UK). The women returned to the unit for a pregnancy test (serum hCG) 14 days after the embryo transfer (ET). According to the departmental protocol, if there was no bleeding and serum levels of hCG was > 40 U/L, the patients were recalled 3 weeks later for an ultrasonography scan (i.e. 5 weeks post OR≈ 7 weeks of gestation) to confirm cardiac activity. Otherwise, if initial levels of hCG were suboptimal or signs of bleeding exist, serial transvaginal ultrasound examinations along with hCG testing were performed earlier, until a normal or abnormal pregnancy was confirmed.

Day 14 post-OR correspond to 25-26 days of conception or the fourth week of gestation because, by convention the 2 weeks of the proliferative phase before ovulation is included when calculating gestation age [219].

4.2.4 Sample collection

The serum samples used in this study were collected from January- December 2005 in conjunction with another prospective study. These samples were taken when the patients came on Day 14 of oocyte recovery (OR) for pregnancy test. This corresponded to Day 12 post embryo transfer (ET) for D2 transfers and Day 11 post ET for D3 transfers). [For details see Sections 2.1.3 and 2.1.4].

4.2.5 Data collection

Baseline data collected from anonymised patient records included: age, cause of infertility, assisted reproductive technology/treatment received, number of embryos transferred, and the Day 2 or 3 of embryo transfer in relation to oocyte retrieval. The outcome records included: Day 14 post OR pregnancy test results, U/S findings at 5 weeks post OR (~ 7 weeks of gestation), gestational week of miscarriage, weeks at delivery, sex, weight, and evidence of structural abnormality of newborn. [See Appendix for data collection forms.].

The clinical utility of HhCG hormone to discriminate between viable pregnancies (containing singletons and twins) and nonviable pregnancies (containing biochemical pregnancies and spontaneous miscarriages), was further analysed by testing the performance of various cut-off values to maximize sensitivity and specificity using the following definitions [218] [For details see Section 2.6]

4.2.6 Pregnancy outcomes

Pregnancy test results in the ACU were obtained using the Siemens Immulite immunoassay for hCG [Section 2.3.2].

Pregnancy outcomes were classified into the groups according to pregnancy status (using the DPC immulite assay system): the non-pregnant (pregnancy test negative, hCG: <5 IU) and the pregnant group (PT positive, hCG: ≥5 IU). In the Edinburgh ACU, a positive pregnancy test was followed by the confirmation of presence of a viable pregnancy on transvaginal ultrasound 5 weeks after the OR (or 3 weeks after the 1st positive pregnancy test), provided that the patient was not bleeding. Patients with suboptimal hCG results (<30 U/L) as well as patients with bleeding underwent further repeated hCG tests and were monitored closely, until ectopic pregnancy was ruled out or hCG levels fell to below 5 U/L.

The pregnant group were primarily classified into the following diagnostic groups:

Viable pregnancy; defined according to current World Health Organization (WHO) definition of childbirth as a pregnancy resulting in delivery of at least one live fetus at >24 weeks gestation, and included singleton as well as multiple pregnancies.

Non-viable pregnancies constituted biochemical pregnancies (defined as a temporary rise in serum beta hCG, along with the absence of signs of intra- and extra-uterine pregnancy by transvaginal ultrasonography) and miscarriages (defined as cessation of development of intrauterine pregnancy seen in TVUS).

4.3 Results for the Edinburgh cohort

4.3.1 Comparison of the pregnant and non-pregnant groups

69 out of 128 women (54%) became pregnant following the IVF/ICSI treatment (hCG test ≥5 IU). There were no differences in the age, aetiology of infertility, type of treatment given, and day of the embryo transfer (ET) between the two groups. However, a statistically significant difference (p=0.004 using Fisher's exact test) was only found between embryo type and pregnancy outcome, since none of the women with frozen embryo replacement became pregnant (Table 26).

Characteristics	Non-pregnant (n=59)	Pregnant (n=69)	P value
Age, mean±SD	36.02 ± 4.32	34.81 ± 3.90	0.102
Aetiology of infertility, n (%)			0.204
• **Male factor**	21 (35.5%)	33 (47%)	
• **Unexplained**	25 (42.3%)	23 (33.3%)	
• **Tubal**	10 (17%)	8 (11.5%)	
• **Endometriosis**	6 (10%)	2 (2.8%)	
• **Mixed**	8 (13%)	7 (10%)	
Type of treatment, n (%)			0.940
• **IVF**	38 (64%)	44 (64%)	
• **ICSI**	21 (36%)	25 (36%)	
Type of Embryo			0.004
• **Fresh**	52 (88%)	69 (100%)	
• **Frozen**	7 (12%)	0	
Day of Embryo transfer (Fresh cycles only)			
• **D2**	34 (57.6%)	39 (56%)	0.324
• **D3**	18 (30%)	30	

Table 26 Paired comparison of pregnant and non pregnant IVF patients showing a statistically significant difference between the groups only with fresh and frozen embryo transfers.

Characteristics	Single embryo transfer (n= 10)	2 embryo transfer (n=118)	P value
Age, mean±SD	37.2 ± 2.1	35.2 ± 4.2	0.023 N.S.
Aetiology of infertility			
• **Tubal factor**	1	9	
• **Endometriosis**	0	5	
• **Male factor**	3	44	0.670
• **Unexplained**	2	38	N.S.
• **Others**	3	6	
• **Mixed**	0	15	
Type of treatment			
• **IVF**	7	75	0.684
• **ICSI**	3	43	N.S.
Type of Embryo			
• **Fresh**	9	112	0.442
• **Frozen**	1	6	N.S.
Pregnancy outcome			
• **Pregnant**	5	54	0.796
• **Non-pregnant**	5	64	N.S.

Table 27 Comparison of the single vs. two embryo transfer groups

There were also no statistical differences in the age, aetiology of infertility, type of treatment given, the embryo type, and the pregnancy outcome between the single embryo transfer and 2 embryo transfer groups (Table 27).

4.3.2 Levels of hCG analytes in viable and non viable pregnancy

Amongst the 69 pregnant women who received fresh embryo transfers, 45 pregnancies (65%) were viable and 24 (35%) were nonviable. The viable pregnancy group included 38 (55%) singleton and 7 (10%) twin pregnancies, whereas the nonviable pregnancy group comprised 15 (22%) biochemical pregnancies and 9 (13%) 1st trimester spontaneous miscarriages (mean gestational age= 10 weeks).

In the viable pregnancy group, 89 % (n= 40) delivered at term (≥37 weeks of gestation), and 11% delivered preterm (n=5) of which, 3 were twin pregnancies. Mean gestational age at delivery in singleton pregnancies was 39 weeks + 6 days and mean birth weight was 3338 grams, whereas for twin pregnancies, the mean gestational age of delivery and birth weight were 36 weeks and 2282 grams, respectively. No information on chromosomal abnormality was available; however, evidence of structural abnormality was reported in two infants (one with pyloric stenosis along with hypospadiasis, and one with extra digits on both hands). There were two neonatal deaths; one was associated with prematurity (spontaneous preterm at 25 weeks) and the cause of second was unknown.

As seen in Table 28, the median (range) concentrations of HhCG, hCG, and hCGβ in viable pregnancies on 14 days post-OR were 374 (81- 1120), 334 (108.5- 991), and 5 (1.8-26) pmol/L respectively, and 131 (23-350), 90 (13-313), and 1.4 (0.2-5) pmol/L in non-viable pregnancies, respectively (Table 29). There was a statistically significant difference between the concentrations of all three hCG in the non-viable vs. the viable pregnancy group ($P < 0.001$) (Table 30, Figures 54-56). The concentrations of HhCG (median with range) for the four groups including biochemical pregnancies, spontaneous miscarriages, viable singletons and viable twins were 49 (23- 191), 207 (100.5- 350), 368 (81- 1120), and 452 (226-619) pmol/L respectively, with the lowest values seen in biochemical pregnancies (Table 31, Figure 54) . The levels of hCG and hCGβ amongst these groups are shown in Table 31 and Figures 55 and 56. Although the average hCG values in twin pregnancies was higher than those in singleton pregnancies, however the difference was not statistically significant.

Total hCG immunoreactivity (HhCG + hCG + hCGβ) showed large variations in the viable as well as the nonviable pregnancy group (ranges of 1.3 to 1.7 and 0.8 to 1.5 respectively) with no statistical significant difference found between the two groups (median= 1.2 in both groups). The ratios of hCGβ to total hCG, and HhCG to hCGβ were similar in both groups (Tables 28-30). This clearly indicates the predominance of HhCG production, compared to other hCG molecular forms, in the period of Day 14 post-OR (Figure 54-59).

Hormone levels in the viable pregnancy group	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum
HhCG	44	1	411.8	203.1	**81.2**	248.4	**374.2**	513.6	**1120.4**
hCG	45	0	338.8	169.2	**108.5**	197.4	**334.4**	405.1	**990.9**
hCGβ	45	0	6.137	4.757	**1.88**	3.381	**5.016**	6.589	**25.803**
HhCG/(hCGβ + hCG)	44	1	1.2231	0.2716	**0.1385**	1.0652	**1.2118**	1.388	**1.7863**
HhCG/hCGβ	44	1	78.83	30.41	**4.5**	61.12	**75.51**	98.58	**150.39**
hCGβ/(hCGβ + hCG)	45	0	0.017708	0.006629	**0.007433**	0.013639	**0.016605**	0.020609	**0.038973**

Table 28 Descriptive statistics for singleton and twins combined in the viable pregnancy group (median and range are highlighted).

Hormone levels of the nonviable pregnancy group	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum
HhCG	23	1	136.9	100.1	**22.8**	36.5	**131**	191	**349.8**
hCG	24	0	110.2	89.2	**13**	26.2	**89.7**	165.9	**313.2**
hCGβ	24	0	1.932	1.393	**0.21**	0.941	**1.493**	3.007	**4.876**
HhCG/(hCGβ + hCG)	23	1	1.2156	0.1712	**0.8038**	1.1054	**1.2112**	1.3675	**1.5215**
HhCG/hCGβ	23	1	73.62	33.93	**28.12**	49.08	**69.96**	93.02	**173.87**
hCGβ/(hCGβ + hCG)	24	0	0.02146	0.01167	**0.00462**	0.01332	**0.01732**	0.02574	**0.04918**

Table 29 Descriptive statistics for biochemical pregnancies and spontaneous miscarriages combined in the nonviable pregnancy group (median and range are highlighted).

	Not Viable		Viable		95% CI for difference	P-value
	N	Median	N	Median		
HhCG	23	131	44	374.2	(179.8- 329.4)	<0.001
hCG	24	89.7	45	334.4	(149.4- 270.3)	<0.001
hCGβ	24	1.49	45	5.02	(2.26- 4.2)	<0.001
HhCG/(hCGβ + hCG)	23	1.21	44	1.21	(0.10- 0.12)	0.974
HhCG/hCGβ	23	69.96	44	75.51	(8.8- 23.1)	0.409
hCGβ/(hCG+hCGβ)	24	0.02	45	0.02	(0.006- 0.002)	0.431

Table 30. Mann-Whitney test results showing differences in the various hCG levels and their ratios between viable and non-viable pregnancies.

	Biochemical Pregnancy (n=15)	Spontaneous miscarriage (n=9)	Singletons (n=38)	Twins (n=7)
HhCG pmol/L	49 (23- 191)	207 (100.5- 350)	368 (81- 1120)	452 (226-619)
hCG pmol/L	39 (13-159)	181 (86- 313)	301 (108.5- 991)	364 (170-525)
hCGβ pmol/L	1 (0.2- 5)	3 (1.5- 5)	5 (2- 25)	6 (3.5- 10)

Table 31 Serum concentrations (median and range) of HhCG, hCG and hCGβ, on Day 14 post-oocyte retrieval.

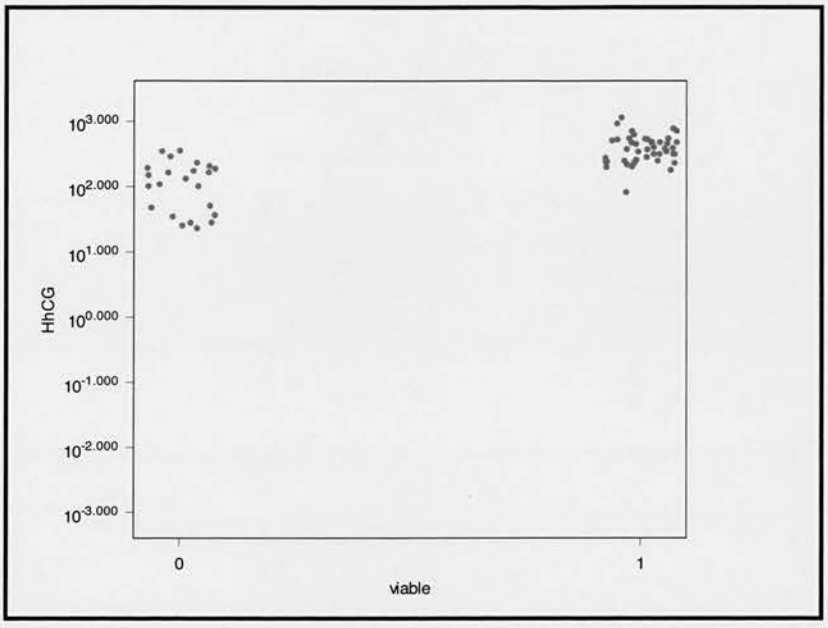

Figure 54 HhCG levels in non-viable and viable pregnancies [0 =not a viable pregnancy consisting of biochemical pregnancy and miscarriage, 1= viable pregnancy consisting of singletons and twins], P value= Significant ($P < 0.001$).

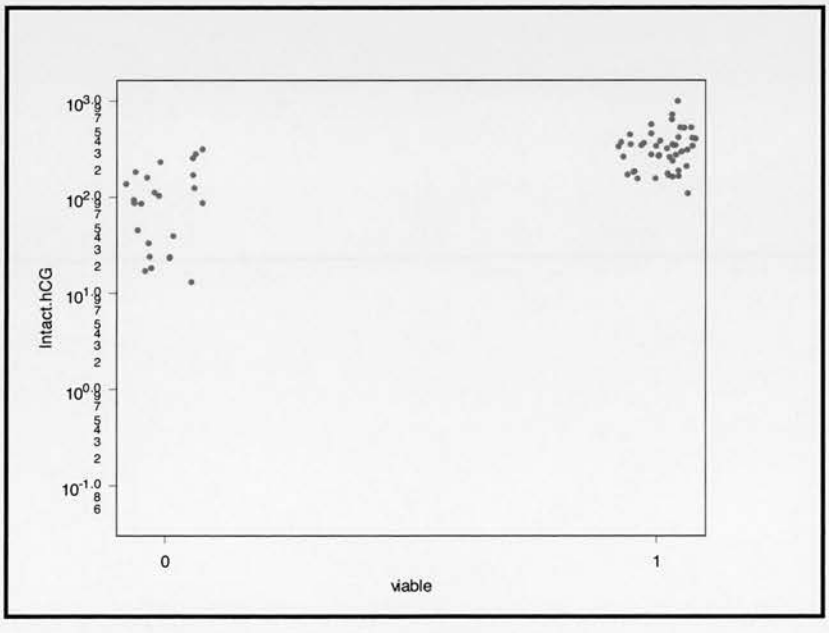

Figure 55 HCG levels in non-viable and viable pregnancies (0= not a viable pregnancy consisting of biochemical pregnancy and miscarriage, 1= viable pregnancy consisting of singletons and twins. P value= Significant ($P < 0.001$).

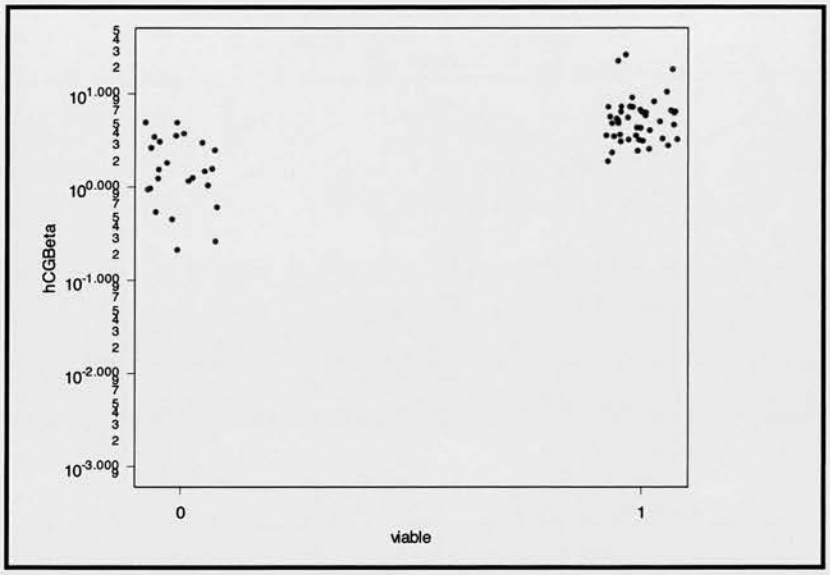

Figure 56 hCGβ levels in non-viable and viable pregnancies (0 =not a viable pregnancy consisting of biochemical pregnancy and miscarriage, 1= viable pregnancy consisting of singletons and twins). P value= Significant (P < 0.001).

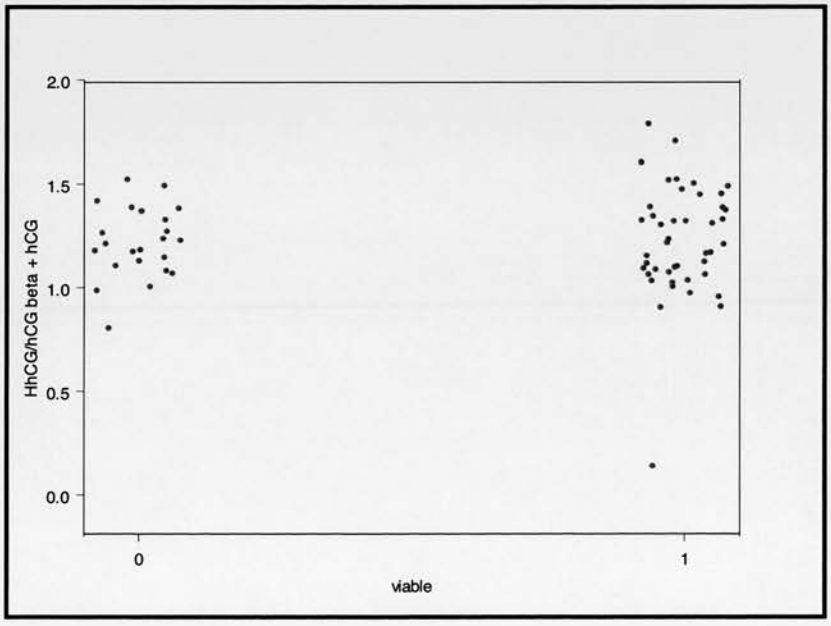

Figure 57 Unaltered ratio of HhCG to total hCG in non-viable vs. viable pregnancies, P-value= NS

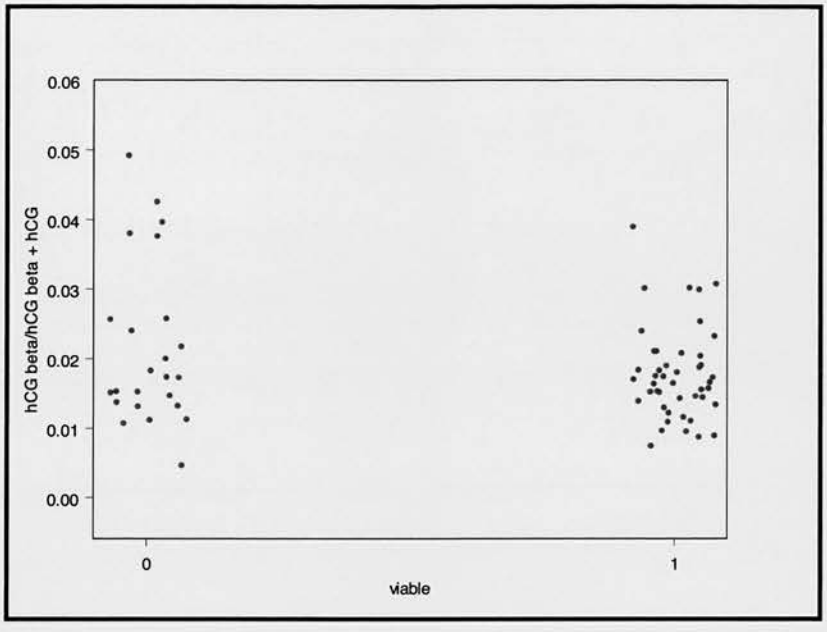

Figure 58 Unaltered ratio of hCGβ to total hCG in non-viable vs. viable pregnancies, P-value= NS

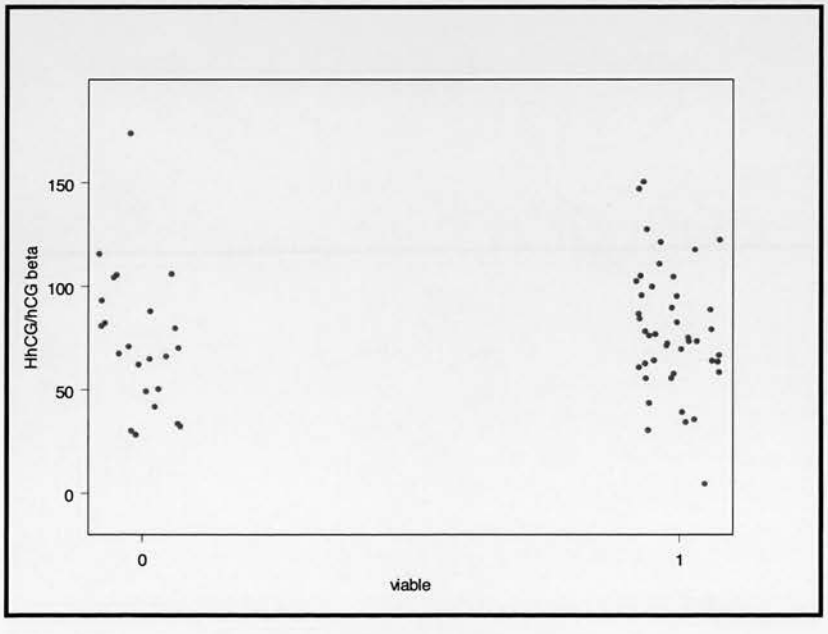

Figure 59 Unaltered ratio of HhCG to hCGβ in non-viable vs. viable pregnancies, P-value= NS

4.3.3 hCG analytes at the day of embryo transfer in viable pregnancies

Mann-Whitney tests have been used to determine whether differences in the level of any of the hormones exist between Day 2 and Day 3 embryo transfers. As seen in Table 32, none of the hCG analytes showed any statistically significant difference in their concentrations or their ratios between D2 to D3 embryo transfers. However only the ratio of HhCG to hCGβ was altered (median on D2= 73.3 and D3= 86.5) indicating that the production of HhCG from the cytotrophoblast is more rapid than the hCGβ production from the syncytiotrophoblast.

Molecule	ET day 2		ET day 3		95% CI for difference	P-value
	N	Median	N	Median		
hCG	26	336.8	19	317.3	(-42.1, 130.5)	0.513
hCGβ	26	5.4	19	4.61	(-0.17, 2.76)	0.087
HhCG	25	368	19	396.5	(-104.3, 112.1)	0.962
HhCG/(hCGβ + hCG)	25	1.15	19	1.32	(-0.29, 0.01)	0.084
HhCG/hCGβ	25	73.3	19	86.56	(-35.67, -1.45)	0.042
hCGβ/(hCG+hCGβ)	26	0.02	19	0.02	(-0.001, 0.007)	0.110

Table 32. Comparison of serum levels of three hormones and their ratios on Day 2 following embryo transfer (ET) vs. Day 3 following embryo transfer. P-value only statistically significant for the HhCG/hCGβ.

4.3.4 ROC analysis

ROC curves plotted the sensitivity of HhCG and other hCG molecular forms measured on the 14th post oocyte retrieval day vs. 1-specificity for a series of multiple cut-off points. The AUC from the ROC curves were compared for HhCG, hCG, and hCGβ, to establish their diagnostic power in predicting the pregnancy outcome amongst the IVF group. The performance of total hCG (pregnancy test positive results obtained with the Siemens Immulite method) was also assessed, since this assay is routinely used for pregnancy testing in the IVF population in the Edinburgh Assisted Conception Unit. The model created by Duc et al. [217] was used to rank the accuracy of a diagnostic test in in this population. [See Section 2.6.2 for details.]

In this study the significance of HhCG levels on Day 14 after the OR in predicting viable from nonviable pregnancies amongst the IVF population was explored using ROC analysis (Figure 60). According to the AUC, HhCG measurement was better than other hCG molecular forms, including total hCG, in distinguishing viable from nonviable pregnancies. However this observation was not statistically significant

(Table 33). All four hCG analytes showed excellent diagnostic accuracy for predicting the ongoing pregnancies (> 90%), but HhCG performed the best.

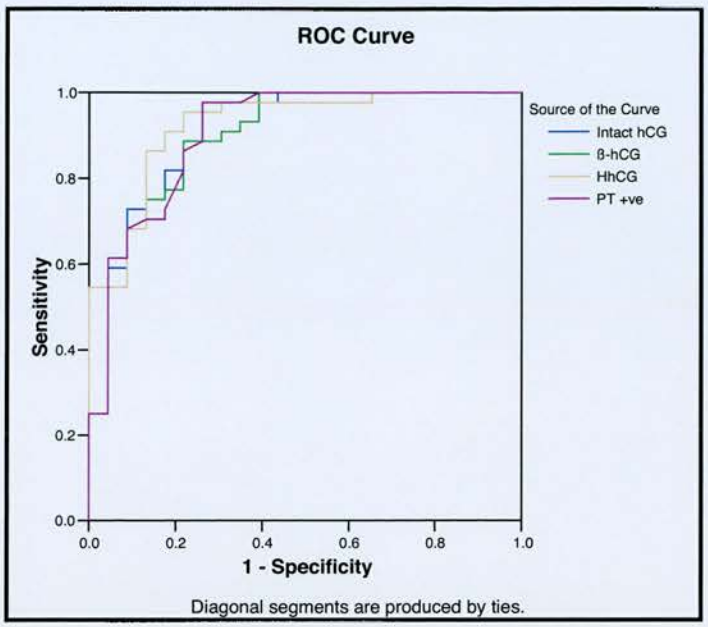

Figure 60 Receiver operator curve analysis for four hCG parameters, including HhCG (yellow line), to assess their diagnostic ability to distinguish viable and nonviable pregnancy.

Test Result Variable(s)	AUC	Std. Error	95% CI	Diagnostic accuracy (DA)
HhCG	.925	.034	.859-.991	++
hCG	.922	.033	.857-.987	++
hCGβ,	.909	.036	.839-.980	++
Pregnancy test +ve	.907	.041	.826-.987	++

Table 33 AUC: area under the curve, DA: diagnostic accuracy. ++ (Excellent)

A single cut-off point of 200 pmol/L for HhCG, based on the AUC of 0.925 (95% CI, .859-.991) was most suitable for predicting viable from the nonviable pregnancy with 93% sensitivity, 80% specificity, 79% PPV (i.e. 55.5% of miscarriages and 100% of biochemical pregnancies), a false-positive rate of 7% and a false-negative rate of 20%. For hCG, the best cut-off level was 165 pmol/L (≈ 56 IU) with 90% sensitivity, 74% specificity, 75% of PPV (55.5% of miscarriages and 86.6% of biochemical pregnancies), false-positive rate of 11%, and a false-negative rate of 25%.

Management of patients with low serum hCG levels (hCG <30 U/L at the Edinburgh ACU) requires repeat hCG testing. In the current IVF cohort, total hCG concentrations of 40 U/L had 100% sensitivity, but with half the specificity of HhCG

(i.e. 40%). While the levels of > 40 U/L detected the entire viable pregnancy group correctly, its PPV was significantly lower (49%) meaning that it is a good predictor of ongoing pregnancy, but fails to efficiently identify women who are at risk of spontaneous miscarriage. In comparison to HhCG, the detection rate for biochemical pregnancy and spontaneous miscarriages was 66.6% and 11%, respectively.

Overall, these results suggest that a single Day 14 post OR level of HhCG is not only diagnostic of pregnancy but also has good predictive value for pregnancy outcome when compared with other serum hCG molecular forms.

4.3.5 Comparison of HhCG results from two methods

Similar to the observation in the UAE cohort, the poor between assay comparability are likely to be related to calibration (Figure 61).

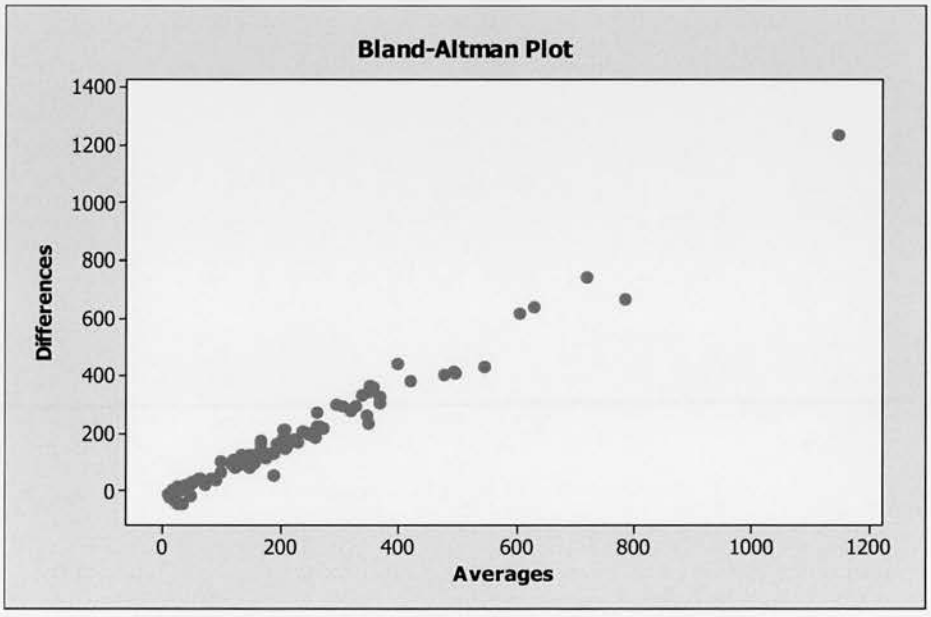

Figure 61 Bland-Altman plot comparing serum HhCG results obtained in the Perkin-Elmer DELFIA method and the Quest Diagnostics method.

4.4 Discussion of results for the Edinburgh cohort

4.4.1 HhCG levels in various pregnancy outcomes

Previous studies have shown that hCG can be detectable in serum as early as 8-9 days post-gonadotrophin surge [492, 493]. There is also evidence of hCG secretion from the embryo affecting the ovarian steroid secretion as early as 6 days following the gonadotrophin surge, [346, 492, 494]. However, hCG is not found in the maternal circulation until there is direct contact and adhesion between the blastocyst and the

endometrium. Hence, it is unlikely that transferred embryos would produce a significant rise of hCG in the maternal circulation within a week, and any hCG-like activity that is detected earlier is likely to be of pituitary origin or from the injected hCG which is given as luteal support to women undergoing assisted conception [95, 120, 495-497]. HCG has been used as a surrogate LH surge due to the degree of homology between the two hormones, and its biological activity and longer circulating half life due to the abundance of sialic acid content [498]. The administration of hCG at the end of the stimulation phase with gonadotrophins triggers the maturation of the oocyte, the resumption of meiosis and the expansion of the cumulus-oocyte complex [112]. Mannaerts et al. showed that 8 days after Pregnyl administration, serum hCG levels had dropped to 10 U/L and when serum samples were tested for hCG on day 15 after oocyte retrieval (day 17 after the Pregnyl injection), hCG values of greater than 5 U/L were regarded as evidence of an invading trophoblast [497]. A large study by Snider et al showed that the 97.5th percentile of nonpregnant women of child-bearing age (18–40 years) had hCG levels of 2.5 U/L (i.e. only one woman had an hCG of 4.6 U/L) and the other cases had undetectable hCG (<2 U/L), clearly emphasising the long-used practice of taking 5 U/L hCG as a cutoff for the detection of pregnancy. However, they suggested that caution must be applied in interpreting a positive hCG result in peri-menopausal women (41–55 years) among whom "background" hCG ranged from 2- 7.7 U/L. They concluded that serum FSH measurement should be incorporated in this group of women to assist in the interpretation of a positive hCG result, as their data showed that pregnancy was unlikely in perimenopausal women with an hCG between 5.0 and 14.0 U/L if the serum FSH was >20.0 U/L. An earlier study by Alfthan et al. confirmed the higher upper limit of serum hCG in nonpregnant peri- and postmenopausal women. Knowledge of the upper limit of "background" hCG is relevant to the assisted conception population since increasing number of older women are opting for it. From all of the above it can be seen that low positive levels in the hCG test, in the IVF population poses a dilemma in terms of interpretation, as well as introduces uncertainty as to the earliest day on which a pregnancy test can be performed. Also, management of patients with suboptimal hCG levels involves several follow-up visits for repeated hCG tests which is time consuming and not cost-effective, and also places extra psychological pressures on an already vulnerable group. In the Edinburgh Assisted Conception Unit, it is standard practice to perform hCG testing, on Day 14 following the oocyte retrieval. Women with serum hCG < 40

U/L have to undergo repeated serum hCG tests until an optimal rise in hCG is seen and pregnancy is confirmed by the transvaginal ultrasound, or until serum hCG levels fall to ≤5 U/L. A similar management practice is followed in many other centres. A psychological study comparing functioning in women at different stages of the in vitro fertilization treatment showed that hostility, depression, and state anxiety scores were highest at the stage of the pregnancy test [491]. Thus, an early marker that can accurately distinguish viable from non-viable pregnancies before the verification of live intrauterine pregnancy by the transvaginal sonography, and that would minimize the need for repeated follow-up, is highly desirable for the IVF population.

HhCG is exclusively produced by phenotypically invasive cytotrophoblast cells which are active at the time of implantation (and in invasive trophoblastic and non-trophoblastic tumors) [70, 72, 74, 175]. There is no evidence of "background HhCG" from the pituitary in nonpregnant women. Hence, alteration in hCG levels after ovarian hyperstimulation and the manipulation of the pituitary-ovarian axis by GnRH analogues and gonadotrophins is unlikely, making hCG a superior candidate for pregnancy testing in an IVF population, compared to other hCG forms. Differential expression of a number of hCG molecular forms, in urine as well as in serum which carry to term and those destined for early pregnancy loss, has been well documented in naturally conceived pregnancies [65, 268, 499] and in the IVF population [66, 67]. Early reports of changes in the temporal pattern of HhCG in continuing and failing pregnancies in an IVF population was achieved using the ratio of hCG concentrations measured by two radiolabelled assays; one with affinity for early pregnancy hCG (B152-B207), asterisk means and the other (B109-B108) with affinity for the later molecular forms of hCG [67]. While the apparent values of hCG (normalized to creatinine) in the B152 assay (i.e. recognizing mainly HhCG) were higher than in the B109 assay in both IVF normal pregnancy and IVF losses, the proportion of each isoform in relation to other (B152/B109 ratio) changed significantly with gestational age and pregnancy outcome. Kovalevskaya et al. assayed the urine samples from the day of embryo transfer until day 20 (1-4 weeks post ET) and found that the B152-recognized molecular forms were first to appear on day 6.75 (range 5-20) from ET, and there was a significant difference in B152/B109 ratio on Day 5-10 and Day 10-15 post ET between normal IVF pregnancy (single fetus) and IVF EPL [67]. The data suggested that HhCG accounts for the major proportion of total hCG immunoreactivity during implantation at the time of trophoblast invasion and the

weeks following it, whilst lower levels were associated with pregnancy losses. However no parameters or cut-off values were described that would differentiate between favourable and unfavourable IVF outcome. Using the same assay methods in the spontaneous conceptions group, they showed that the B152 antibody was able to detect 81% of clinical pregnancies and 36% of early pregnancy losses cycles (EPL) [65]. The corresponding figures for the molecular forms recognized by the B109 antibody were 58 and 36%, respectively. The current study is among the first to evaluate the usefulness of quantitative HhCG levels for clinical application in the prediction of pregnancy outcome in an IVF population, by assessing the sensitivity, specificity, and positive predictive value of the test. The present prospective study also included measurements of hCG and hCGβ, allowing us to directly compare the three hormonal parameters in terms of their diagnostic accuracy in the prediction of IVF outcome. Our results show that the concentration of HhCG was significantly higher on day 14 post oocyte retrieval (OR) for viable pregnancies (i.e. singletons and twins) compared to nonviable ones (i.e. biochemical pregnancies as well as spontaneous miscarriages) confirming the findings of Kovalevskaya et al. [66, 67] (Figures 54 and 55 and Tables 29-31). The concentration of HhCG in the nonviable group varied from 23 to 350 pmol/L (median= 131 pmol/L). This was lower than the variation found in the viable group (i.e. range 81- 1120 pmol/L, median= 374 pmol/L). The finding of lower levels of HhCG in failing pregnancies has also been documented in naturally conceived pregnancies in many reports [65, 81] and is consistent with the present results from the UAE cohort [Section 3.7]. Similarly, the levels of hCG and hCGβ in the viable pregnancy group were higher than in the nonviable group, ranging from 108.5 to 991 pmol/L (median= 334 pmol/L) and 2 to 26 pmol/L (median= 5 pmol/L), respectively (Figure 55 and 56 and Tables 28-31). This is also in agreement with many other previous studies [218, 424, 485, 486, 500-503].

As seen from Tables 28-30 and Figure 57, 100% of total hCG immunoreactivity in day 14 post-OR serum samples was HhCG (HhCG/ hCG + hCG β, %) in both viable and nonviable pregnancies. No difference in this ratio was observed between the two groups (median of 1.2 in both), indicating the dominance of HhCG production from the cytotrophoblast during the very early period of gestation, irrespective of the pregnancy outcome. The ratio of hCGβ/total hCG, and HhCG/ hCGβ also remained unchanged (Figures 58 and 59 and Table 28-30,). This is not surprising, considering the dominance of HhCG-secreting cytotrophoblast cells during the early stages of

blastocyst differentiation. When cytotrophoblast cells fuse to form intermediate cells, then syncytiotrophoblasts, the proportions of HhCG rapidly fall concurrently with advancing gestation, as shown in the UAE cohort of this study and by many other investigators [66, 67, 70, 81]. Indeed, it was previously shown that the shift in the relative proportion of HhCG in relation to later hCG molecular forms as the gestation progresses had a better power of discriminating between the continuing and non-continuing IVF pregnancy groups than when the time-frame shift was eliminated, suggesting that as gestation proceeds the biological activities of cellular factors controlling the expression of hCG molecular forms and cytotrophoblast differentiation become apparent in both continuing as well as in failing-pregnancies [66, 67]. A later study further emphasized that using proportion of HhCG at the time of implantation was a stronger discriminator of failing and continuing pregnancies compared to the HhCG or hCG alone [504].

As discussed in previous chapters, serial measurements of serum hCG during early gestation are critical for the evaluation and differentiation of normal from abnormal pregnancies. Similar to the pattern of hCG rise in spontaneous pregnancies described by Kadar et al. and Barnhart et al. [220, 505], several authors have tried to characterize the pattern of serial hCG rise in an IVF cohort [487, 506, 507]. A mean hCG doubling time of 1.59 days for days 12–16 post-OR was shown by Richards et al. [485], 1.57 days for days 11–23 post-OR by Zegers-Hochschild et al. [508] and 1.6 days at day 12 to about 3 days at 24 days after ET by Stone et al. [507]. Also in most studies, the curve was shown to be quadratic with an earlier plateau (i.e. the rate of increase slows at ~24 days post-oocyte retrieval, or a gestational age of 5 weeks and 3 days) but on average, an increase of 50% in 1 day and 124% in 2 days is generally seen [509]. The relatively early plateau in hCG production may reflect differential rates of implantation of multiple embryos transferred or resorption of non-viable embryos. Alternatively, it is possible that the administration of exogenous progesterone given for luteal support, leads to a diminished requirement for hCG-induced steroidogenesis by the corpora lutea [509].

Although the absolute hCG values are higher in multiple pregnancies, than in singleton pregnancies, no difference in doubling time has been shown [508, 509]. However, data on hCG doubling time in miscarriages and ectopic pregnancies have been conflicting. While some reported a slow rise of hCG (or delayed DT) [248, 508], others reported a normal rise and doubling time [510]. In both cases, the pattern of hCG rise has been shown to be a poor indicator in discriminating miscarriages from ectopic

pregnancies and it has been suggested that the rate of rise is dependent on many factors such as multiple embryo transfer leading to multiple gestations, BMI, and the syndrome of vanishing twins [487, 488, 509, 511]. Indeed, studies have shown that even second-trimester maternal serum levels of hCG, AFP and unconjugated oestriol are altered in pregnancies conceived by conventional IVF or ICSI [512-514]. The false positive rate in Down syndrome screening in these groups is found to be higher compared to spontaneous pregnancies. The reason for level alterations in these markers in pregnancies achieved through assisted reproduction remains unknown. The fact that the median age of women becoming pregnant through assisted reproduction is higher than in spontaneous pregnancies may be one possible reason for higher false positive rates. Alternatively, it has been hypothesized that high hCG levels may be a result of the higher levels of progesterone found in these pregnancies compared to natural conceptions, which may have resulted from ovarian hyperstimulation and the subsequent formation of multiple corpora lutea [514]. However this hypothesis has been disputed since hCG elevation was also observed in women who conceived after ovum donation and frozen embryo transfer, where the possibility of excessive follicles and multiple corpora lutea does not exist [512, 515, 516]. Differences in the growth and development of the fetus and sub-optimal placentation have been suggested an effect of ICSI (embryos produced from ICSI have a tiny hole in the zona pellucida) and freeze-thawing of an embryo [513]. Indeed, many studies (including our own) have shown that the implantation potential of frozen-thawed embryos is lower than that of fresh embryo. However, the reasons why frozen-thawed embryos following implantation have increased hCG production in some studies is as yet unknown. The mechanical and/or temperature related insult on the embryo may trigger a compensatory hyper-reactive secretion by the growing trophoblast, although why the placental markers alter is still not known. However, many authors agree that appropriate adjustment for some variables is necessary in the screening programme for assisted reproduction pregnancies [512-514].

Although the average levels of all hCG forms in multiple pregnancies were higher than in singleton pregnancies in the current study, this difference was not statistically significant (Table 31). This was probably due to the small number of twins along with variations in the day of sample collected (Day 11 or 12 post ET). Performing the study with a larger group may yield significantly higher hCG results for multiple

pregnancies, as has been shown in many previous studies [218, 500, 501, 517], and which represents higher hCG production from multiple implanted embryos.

4.4.2 Levels of hCG by the day of embryo transfer

Some researchers have recommended embryo transfer on Day 5 post-oocyte retrieval (if certain criteria were met) because of the higher reported implantation and pregnancy rates associated with blastocysts with 64+ cells on Day 5 [518], although there is no general agreement in the literature. During the early days of IVF, culture media that were able to sustain embryonic development for days and provide the best environment for the survival of the embryo were lacking, leading to embryos being transferred 2 days following the OR at the 2- to 4-cell stage. The timing of the arrival of the embryo in the uterus during that stage is premature compared with the situation in spontaneous conceptions, where the embryo enters the uterus at the morula stage, 4-5 days after ovulation [183, 519] (Figure 62). Advantages of prolonged embryo culture lie in synchronization between embryos and endometrium, and transfer of embryos to the uterus on Day 3 (or later) mimics more closely the physiologic time of uterine entry than day 2 ET, and also allows better selection of viable embryos [519]. While the ideal time of embryo replacement remains debatable, many prospective and retrospective data show that the mean embryo quality of D2 embryos is slightly better than D3 embryos, and pregnancy and implantation rates are comparable in day 2 and day 3- embryo transfers (or even day 5-6 transfers) [519-521]. One study showed that an hCG test on day 16 post-retrieval after blastocyst transfer a better predictor of clinical outcome than a hCG value after a day 3 embryo transfer, and the probability of an ongoing pregnancy was 90% using a hCG cut-off value of >100 U/L (for D3 post OR), and 97% at a level of >300 U/L (for D5 post OR) [518]. The current study data failed to show any difference in pregnancy rate between the Day 2 (n=26) or Day 3 (n=19) embryo transfers (Table 32), or in any of the hormone levels on Day 14 post OR (a pregnancy test on Day 14 of oocyte recovery for D2 transfers corresponds to Day 12 after ET, whereas for D3 this corresponds to day 11 of ET). However, the ratio of HhCG to hCGβ was significantly altered (P=0.042) (Table 32). An increase in the ratio from D2 to D2 transfers is likely to be due to the dramatic increase in HhCG levels, compared to a slower increase in hCGβ from an embryo which is in a relatively more advanced developmental stage. Also, the higher ratio may reflect a

higher rate of proliferation of cytotrophoblast cells on D3 compared to D2, with less differentiation.

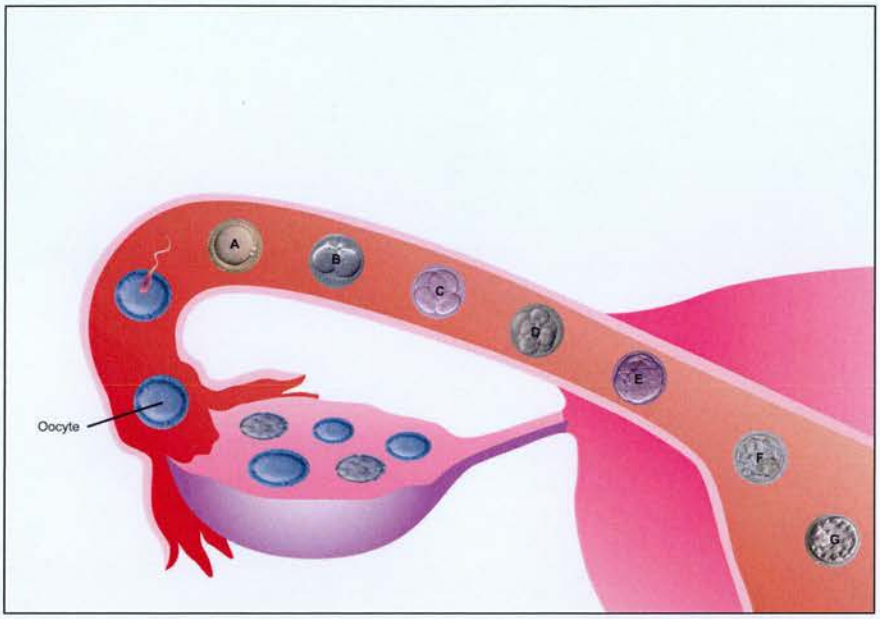

Figure 62 The early stages (shown as A-G above) of human development from fertilization to blastocyst formation. Day 3 ET or later mimics the physiological time of embryo entry into the uterus in spontaneous conceptions. [Figure adapted and extended from Reference 184.]

The number of embryos transferred is also critical to successful IVF/ICSI treatment and many centres try to improve their "take-home-baby rate" per treatment cycle by transferring more embryos. This leads to higher rates of multiple birth, without necessarily improving the overall success rate, since the risk of adverse perinatal outcome associated with multiple pregnancies such as preterm births and low birth weights are higher [522-524]. To reduce the incidence of higher-order multiple gestation, methods such as single embryo transfer and/or transfer of embryo at the blastocyst stage have been suggested [525, 526]. However this is at the expense of reducing the chances of live birth in an IVF cycle [523]

4.4.3 Screening performance of HhCG in the Edinburgh cohort

In selecting one group for closer follow up on the basis of the hCG level measured on Day 12-14 after embryo transfer, two things must be considered before choosing a cut-off value, i.e. the lower the hCG value (lower sensitivity) the higher is the likelihood of non-viable pregnancy (specificity), and the higher the hCG cut-off value, the lower the likely positive predictive value (true spontaneous abortion) as a result of

the increase in sensitivity and the reduction in specificity [218]. Thus, setting a higher cut-off point in order to increase sensitivity should not be a priority in the IVF population, since this would decrease the specificity considerably. Hence, not only will it not discriminate vital from potentially life-threatening ectopic pregnancies, but it will also add to the psychological stress on an already vulnerable population by giving them false reassurance. The same rule applies for conducting a hCG test before Day 12-14 of ET, as the earlier the test is performed, the higher the rate of false-negative tests. Selecting a single cutoff value for HhCG for each day of ET was not possible in this study due to the small sample size of the positive pregnancy test group in this study. Moreover, splitting the outcomes further into Day 11 or 12 of post-ET would yield bias, or to a statistically non-significant result. Hence the diagnostic performance for all hormonal cut-off values was calculated irrespective of the day of ET.

The significance of HhCG levels on Day 14 after OR in predicting viable from nonviable pregnancies in the IVF population by ROC analysis using the model created by Duc et al. [217] to rank the accuracy of a diagnostic test (Figure 60). It was found that according to AUC analyses, HhCG measurement was superior to other hCG molecular forms, including total hCG, in distinguishing viable from nonviable pregnancies. However this observation was not statistically significant (Table 33). All four hCG analytes showed excellent diagnostic accuracy for predicting ongoing pregnancies (> 90%), but HhCG performed best. The cut-off level of 200 pmol/L gave the best balance between sensitivity and specificity, based on an AUC of 0.925 (95% CI, .859-.991), and distinquished viable from nonviable pregnancies with 93% sensitivity, 80% specificity, 79% PPV (i.e. 55.5% of miscarriages and 100% of biochemical pregnancies), a false-positive rate of 7% and a false-negative rate of 20%. In contrast, the cut-off level for hCG (165 pmol/L) (≈ 56 IU) yielded only 90% sensitivity, 74% specificity, 75% of PPV (55.5% of miscarriages and 86.6% of biochemical pregnancies), false-positive rate of 11%, and false-negative rate of 25%. As the sensitivity of both molecules was similar, the choice of superiority of a marker depends on weighing the cost of unnecessary intervention and close monitoring of continuing pregnancies but are falsely identified as a miscarriage or non-continuing pregnancy, against the health risks and hazards of falsely identifying a non-continuing pregnancy as a continuing one. The latter choice of becomes more important in the assisted conception population since pregnancies obtained after an IVF/ICSI and

embryo transfer (ET) are at risk of adverse outcome compared to natural conceptions, including the risk of ectopic pregnancy and spontaneous miscarriages [488, 489]. The superiority of HhCG as a marker is due not only to its excellent diagnostic accuracy for predicting the ongoing pregnancies, but to its higher PPV and lower false-positive and false-negative rates. Even the criterion of our centre to follow up patients with suboptimal levels of hCG (> 40 U/L) has the ability to correctly identify the entire viable pregnancy group. However, with a PPV of 49%, it is a poor predictor of non-continuing pregnancies and ectopics.

Different cutoff levels have been proposed by different authors. Bjercke et al. showed that a cut-off value of 55 U/L had a 90% chance of a vital pregnancy after IVF and embryo transfer, whereas the positive predictive value (i.e. the probability that a patient will have an early pregnancy loss) was 60% [218]. Lambers et al. proposed a cut-off point for Day 14 and 15 of 76 U/L and 142 U/L, respectively [503]. Urbancsek et al. suggested that a cut-off level of 50 U/L would predict pregnancy outcome with a sensitivity of 75% and a specificity of 81%, while an hCG value >135 U/L would predict a multiple ongoing pregnancy with a sensitivity of 80% and a specificity of 88% [501]. Sugantha et al. reported a discriminatory hCG value of 50 U/L for day 14 after oocyte retrieval, with a 79% of sensitivity and 78% specificity [517].

The present study results are in agreement with previous studies showing low levels of HhCG amongst pregnancies destined for failure, and has the ability to accurately identify 100% of biochemical pregnancies, making it the most accurate single early marker of biochemical pregnancy currently available [66, 67, 81]

4.4.4 Limitations of the Edinburgh IVF / ICSI cohort study

The use of a single hCG cutoff value in an IVF setup remains controversial and may not be generally applicable. Although many investigators have attempted to obtain the best possible cutoff value to discriminate viable from non viable pregnancies using hCG immunoassays, the results obtained from these studies are heterogeneous, with differences in the definitions of pregnancy outcome, extrapolation of data, study design, study population, risk factors, timing of hCG tests in relation to the day of embryo transfer, and many other factors which may influence the hCG results in each research study. Studies should include specific inclusion and exclusion criteria to create a more homogeneous sample to determine the prognostic significance of hCG. The IVF cohort studied was not representative of the whole IVF population, since the

rate of non-viable pregnancy which included BP and EP was higher than for the whole IVF cohort. Only patients with known outcomes were included which may have produced an imbalance in the data, and may have been partly due to the criteria used to define non-viable pregnancy. In this regard many studies have shown widely differing incidences of adverse pregnancy outcome based on the definitions used and studies reporting IVF success rates do not use a standardised definition of pregnancy. Other limitations relevant to the spontaneous pregnancy cohort [see Section 3.9] may also be applicable to the assisted conception cohort. These include the following:

1. Variables that influence the IVF outcome (e.g. parity, prior spontaneous abortions, tobacco use, FSH levels, number of IVF cycles) were not assessed.
2. The study included a relatively small sample number and thus had limited power to detect differences in subgroup analysis.
3. Data on the chromosomal abnormalities or any other maternal disease was not available but might also have prognostic significance.
4. There is no International Standard for HhCG which may contribute to the poor between assay comparability observed (Figure 61). Differences in assay protocols may all influence the applicability of our finding in general population.

In summary, discriminatory hCG cutoff values may be useful in predicting implantation outcome in IVF-ET cycles and may help to guide clinicians in identifying pregnancies at risk for adverse outcomes to enable more intensive surveillance of this population. Results suggest that a single Day 14 post OR level of HhCG is not only diagnostic of pregnancy but also has good predictive value for the pregnancy outcome, when compared with other serum hCG molecular forms. This information also may be useful in providing counselling to IVF patients regarding pregnancy prognosis and could also perhaps result in cost savings.

CHAPTER 5. SDS-PAGE AND WESTERN BLOT CHARACTERIZATION OF OLIGOSACCHARIDE HETEROGENEITY IN HCG FROM DIFFERENT SOURCES

5.1 Background to the study of molecular heterogeneity

hCG is a glycoprotein containing N-linked and O-linked oligosaccharides which exhibit high variation in their glycosylation pattern ranging from mono- to multi-branched carbohydrate structures. The carbohydrate content of hCG changes in different physiological and pathological situations. As glycosylation pattern of hCG reflects the physiological or pathological state of trophoblastic cell, it has been extensively investigated for its usefulness in the prediction of pregnancy complication as well as in the diagnosis and monitoring the course of trophoblastic disease.

The purpose of the experiments undertaken here were to investigate the variation in carbohydrate content of hCG purified from different sources including normal pregnancy (Pregnyl) and hCG produced from cancer cell line (JEG3) using MAbs and a panel of commercially available lectins. Qualitative differences in lectin binding affinity for certain sugar residues that discriminate hCG glycoforms in urine samples from women with normal pregnancy and molar pregnancy were also explored in order to evaluate the usefulness of this method in the diagnosis and prognosis of pregnancy complication and trophoblastic diseases.

5.2 Results of studies of molecular heterogeneity

5.2.1 Carbohydrate heterogeneity using different MAbs in hCG from pregnancy sources and JEG3 cell lines

5.2.1.1 Studies with MAb 8F11SMA

Directed to antigenic site of hCG which is available on hCG, hCGβ, and the hCGβcf, including hCG minus the CTP and hCGβ missing the CTP.

Many protein bands at approximately 65, 50, 37, 25 and 23 kDa were identified in non-reduced purified hCG (non-reduced Pregnyl), and additional higher bands of 150 and 96 kDa were detected in the non-reduced choriocarcinoma hCG (non-reduced JEG3) (Figure 63)

After reduction, the purified hCG showed two distinctive bands of 36 and 24 kDa, identified as the β-subunit of hCG and the β-subunit minus the CTP. However, no bands were detected for reduced choriocarcinoma hCG indicating that carbohydrate content of the hormone has altered during its mobility in the SDS-PAGE. Since upon

reduction, the hCG dimer dissociates into its subunits, the earlier 50 and 37 kDa bands detected under the non-reducing conditions can be identified as intact hCG dimer with variation in sialic acid content. (Figure 64.

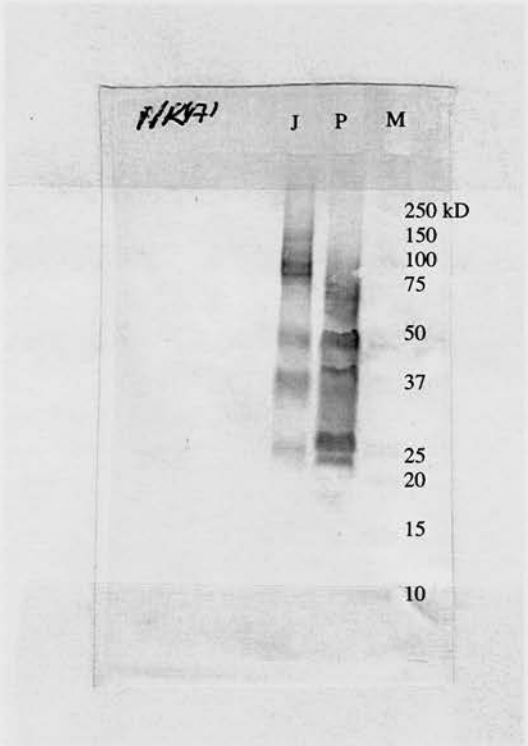

Figure 63 Probing of Pregnyl (P) and JEG3 (J) hCG with MAb 8F11SMA [Non-reducing conditions; molecular weight markers (M) shown].

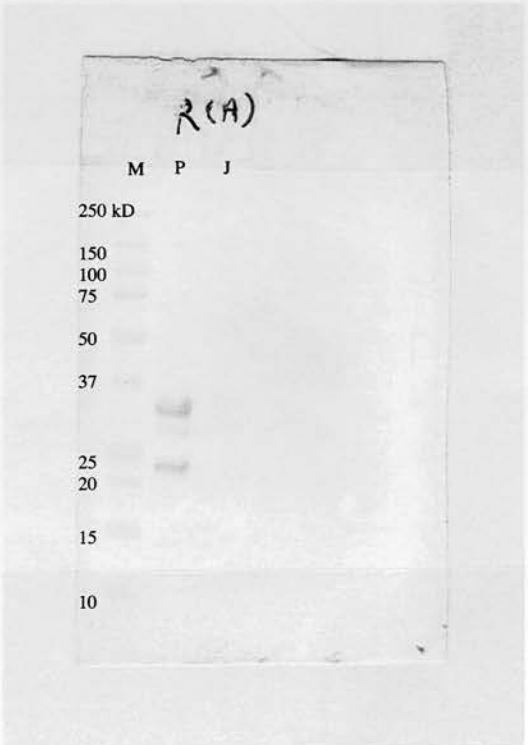

Figure 64 Probing of Pregnyl (P) and JEG3 (J) hCG with MAb 8F11SMA [Reducing conditions; molecular weight markers (M) shown].

5.2.1.2 Studies with MAb B152

Two bands of 46 and 37 kDa were detected in non-reduced Pregnyl as well as non-reduced JEG using the B152 antibody indicating its ability to detect the hCG dimer besides the hyperglycosylated form of hCG present in both purified as well as choriocarcinoma hCG (Figure 65.

Upon reduction, purified hCG dissociated into two bands at about 35 and 30 kDa identified as hCGβ with heterogeneous carbohydrate composition, whereas reduced JEG3-hCG showed bands at 50 and 37 kDa indicating that hCGβ derived from choriocarcinoma cell lines is indeed hyperglycosylated (Figure 66).

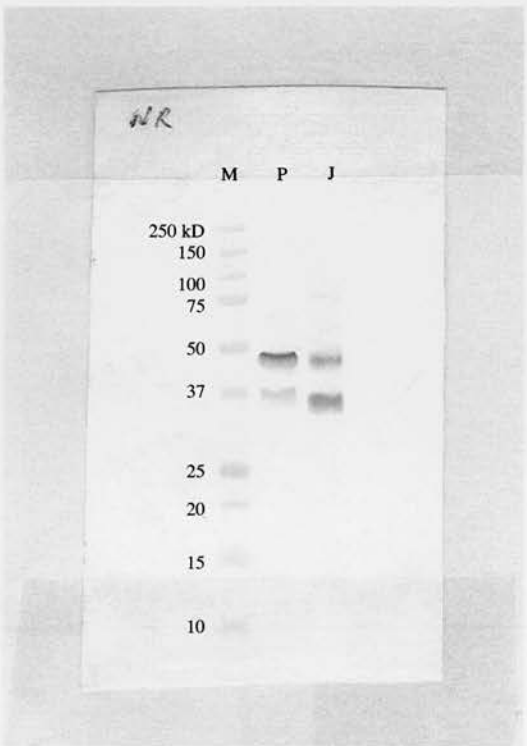

Figure 65 Probing of Pregnyl (P) and JEG3 (J) hCG with MAb B152. [Non-reducing conditions; molecular weight markers (M) shown].

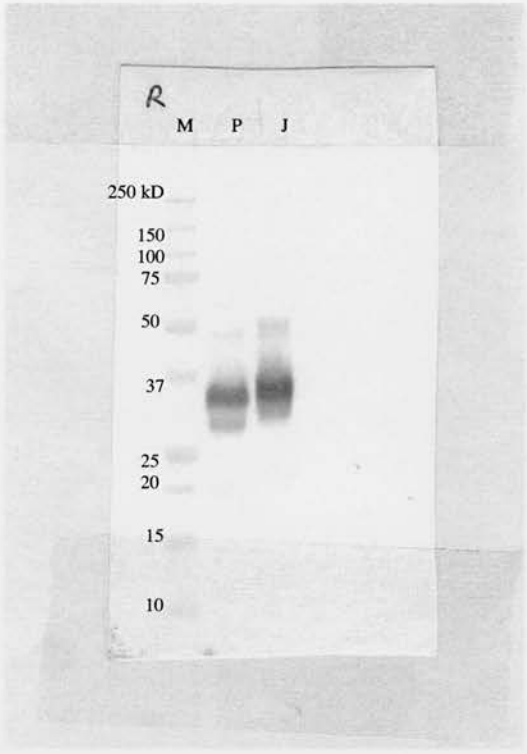

Figure 66 Probing of Pregnyl (P) and JEG3 (J) hCG with MAb B152 [Reducing conditions; molecular weight markers (M) shown].

5.2.2 Examination of carbohydrate heterogeneity of Pregnyl and JEG3 by lectin-binding

Variations in lectin-binding patterns to hCG were seen between the pregnancy sample as well as the hCG derived from JEG3 choriocarcinoma cell line for both non-reduced and reduced forms. More pronounced binding pattern could be demonstrated in reduced purified hCG upon reduction. Results of the lectin-binding are expressed in terms of the intensity of the colored-bands visualized (++ dark band, + light band, and ± faint band). Lectins were classified into four groups according to their broad sugar specificities, i.e. **Group 1 GlcNAc-binding lectins:** WGA, *Solanum tuberosum*, *Griffonia simplicifolia* II, succinylated WGA, *Lycopersicon esculentum, Phaseolus vulgaris* erythroagglutinin); **Group 2 GalNAc-binding lectins**: Jacalin, soya bean agglutinin, *Amaranthus caudatus* lectin, Peanut agglutinin, *Dolichos biflorus* agglutinin, *Saphora japonica* agglutinin *Griffonia simplicifolia* I, *Erythrina cristagalli* lectin, *Vicia villosa* lectin; **Group 3 Mannose/galactose/fucose-binding lectins (neutral sugars)**: *Lens culinaris* agglutinin, Jacalin, Soya bean agglutinin, *Lotus tetragonolobulus* lectin, *Amaranthus caudatus* lectin, *Datura stramonium* agglutinin, peanut agglutinin, Con A, *Ulex europaeus* agglutinin I, Phaseolus vulgaris

leucoagglutinin, *Pisum sativum* agglutinin, *Saphora japonica* agglutinin, *Griffonia simplicifolia* I lectin, *Lathyrus odoratus* lectin, *Erythrina cristagalli* lectin, *Phaseolus vulgaris* erythroagglutinin; **Group 4 Sialic acid-binding lectins:** WGA, succinylated WGA, *Sambucus nigra* agglutinin, *Solanum tuberosum* and *Lens culinaris* agglutinin

5.2.2.1 Lectin binding to Pregnyl

Non-reduced hCG bound to lectins recognizing neutral sugar components of α- and/or β-N-linked chains of hCG (Figures 67 and 68). Reduced hCG bound to all of the lectins except soya bean agglutinin and *Griffonia simplicifolia* I and II (Figures 69 and 70).

5.2.2.2 Lectin binding to JEG3 hCG

Since lectins bound more to purified hCG after reduction, affinity to lectin binding was tested only on the reduced JEG3 hCG forms which showed affinity to either neutral sugar or sialic acid binding (Figures 71 and 72). JEG3 hCG bound to lectins identifying mannose, N-acetylgalactosamine, α-linked L-fucose and N-acetylglucosamine.

From these results, the heterogeneous glycoform patterns of hCG from different sources is evident. Pregnancy hCG appears to more sialylated than the choriocarcinoma hCG. Both *Solanum tuberosum* and *Sambucus nigra* agglutinin showed bands at 20 kD confirming the presence of sialic acid in the hCG-CTP region of hCG from normal pregnancy.

A faint band is also seen at the 36 kD region in Pregnyl with results for the *Sambucus nigra* agglutinin lectin indicating hCGβ is sialylated. In comparison to hCG from Pregnyl, JEG3-hCG appears to be more fucosylated. The fucose-binding lectin *Lotus tetragonolobulus* shows bands in hCG-CTP region and hCGβ, at 25 kD and 36 kD, respectively. Triantennary branching on hCGβ was observed in choriocarcinoma hCG but not normal pregnancy hCG.

No difference in the mannose binding lectins was observed in the hCG preparations from the two sources, lectins recognizing terminal N-acetylgalactosamine and galactose bound more strongly to Pregnyl hCG.

Code	Lectin	Abbrevn	Specificity	Pregnyl	JEG3
2	*Lens culinaris* agglutinin	LCA	α-linked mannose; sialic acid	++(25), +(32), ++(36)	+(25), ++(37)
3	Wheat germ agglutinin	WGA	Trimers and tetramers of N-acetylglucosamine; sialic acid	++(20-25), ++(36)	++(25) ,++(37), ++(75)
4	*Galanthus nivalis* agglutinin	GNA	α-1,3 mannose (Terminal mannose)	++(20)	++(25), +(37), ++(75)
6	Jacalin	J	galactosyl (β-1,3) N-acetylgalactosamine (T-antigen)	++(25), ++(37)	++(37)
7	Soya bean agglutinin	SBA	α- or β-linked N-acetyl-galactosamine; galactose	±(25), ±(36)	+(25), ++(75)
9	*Sambucus nigra* agglutinin	SNA	Sialic acid on terminal galactose α (2-6) > α (2,3) NeuAcα (2–6)Gal/GalNac	+(20), ± (36)	
10	*Lotus tetragonolobulus* lectin	LT	α- or β -linked-fucose containing oligosaccharide		++(25), ++(36)
11	*Amaranthus caudatus* lectin	ACL	Galactosyl (β-1,3) N-acetylgalactosamine (T-antigen)	++(30), ++(37)	++(37)
12	*Solanum tuberosum* lectin	ST	N-acetylglucosamine; N-acetylmuramic acid	++(20)	
14	*Datura stramonium* agglutinin	DSA	Galactosyl (β1-4) N-acetyl glucosamine	+(25), +(30) ++(36)	+(25), ++(37)
15	Peanut agglutinin	PA	Galactosyl (β1-3) N-acetyl galactosamine (T-antigen)		+(25)
18	Concanavalin A lectin	Con A	Mannose-containing N-linked chains (usually α-linked)		++(25), ++(37), ++(75)
19	*Dolichos biflorus* agglutinin	DFA	α-linked N-acetylgalactosamine		
20	*Ulex europaeus* agglutinin I	UEA	Fucosyl α(1,2); galactosyl β(1,4)		
21	*Phaseolus vulgaris* leucoagglutinin	PHA-L	β(1–6) triantennary branching (Ca^{2+} and Mg^{2+} essential)		+(37)
22	*Pisum sativum* agglutinin	PSA	α-linked glucose or mannose (Ca^{2+} and Mg^{2+} essential)	+(25), ++(26), +(35), ++(37)	+(37), +(50)
23	*Saphora japonica* agglutinin	SJA	Terminal N-acetylgalactosamine; galactose (binding enhanced at alkaline pH)	+(25)	
24	*Griffonia simplicifolia* I lectin	GS-I	α-/β-linked N-acetyl-galactosamine; galactose	± (25)	
25	*Phaseolus vulgaris* erythroagglutinin	PHA-E	Galβ(1–4)GlcNacβ(1–2)Man	++(25), ++(37)	++(25), ++(37), ++(50), ++(75)
27	Succinylated wheat germ agglutinin	WGA-S	N-acetylglucosamine	++(25), +(37)	+(25), +(37)
29	*Vicia villosa* lectin	VV	α-/β-linked terminal N-acetylgalactosamine	++(25), +(35), ++(37)	
31	*Lycopersicon esculentum* lectin	LE	Trimers/tetramers of N-acetylglucosamine	++(25), +(37)	+(37)
32	*Erythrina cristagalli* lectin	EC	galactosyl (β-1,4) N-acetylgalactosamine	+(25), +(37)	++(25)
33	*Griffonia simplicifolia* II lectin	GS-II	α-/β-linked N-acetylglucosamine		
34	*Lathyrus odoratus* lectin	LO	mannose	+(22), ++(25), +(35), ++(37)	+(37), +(50)
35	Alkaline phosphatase: streptavidin	Control	Biotin		

Table 34 List of lectins used with their binding specificities and results of lectin-probing in Pregnyl as well as JEG3. (Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuNAc, N-acetylneuraminic acid; Man: mannose).

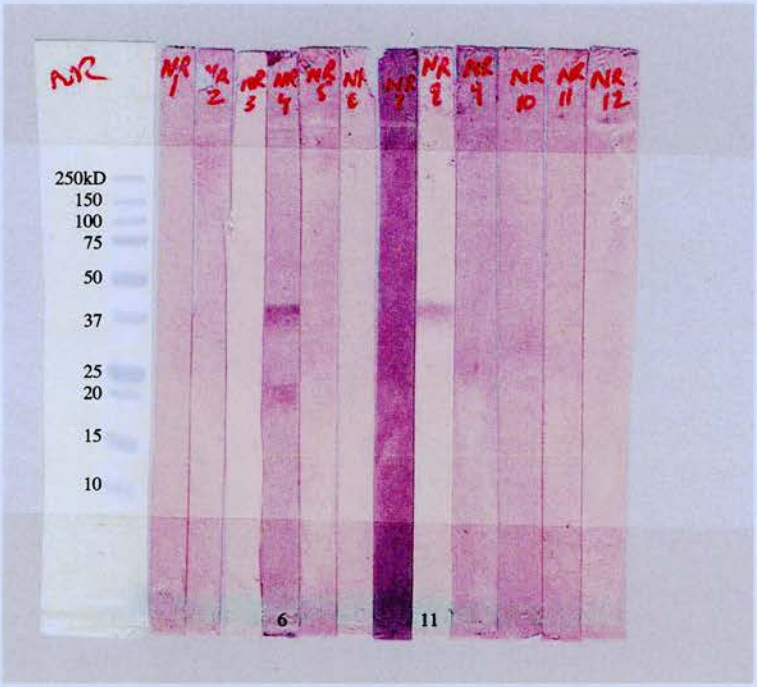

Figure 67 Lectin probing of Pregnyl. For lectin codes see Table 34. [NR, non-reducing conditions; molecular weight markers (M) shown].

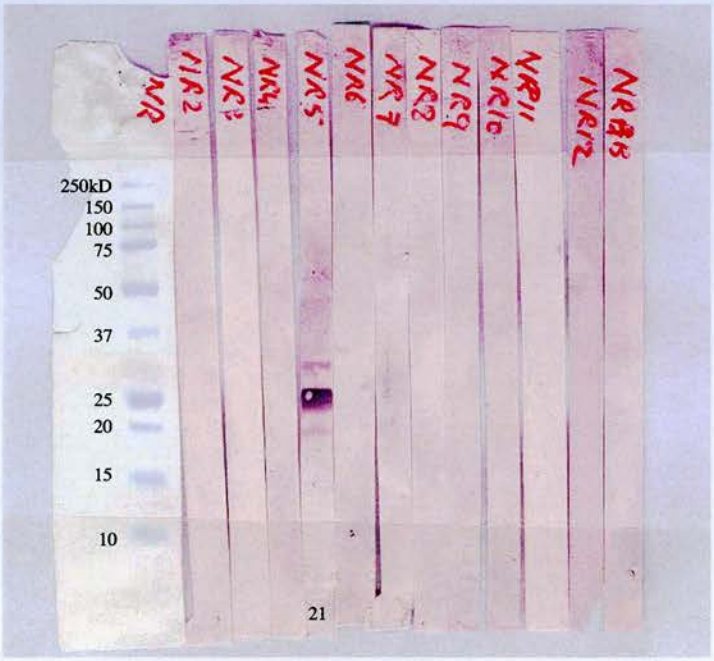

Figure 68 Lectin probing of Pregnyl. For lectin codes see Table 34. [NR, non-reducing conditions; molecular weight markers (M) shown].

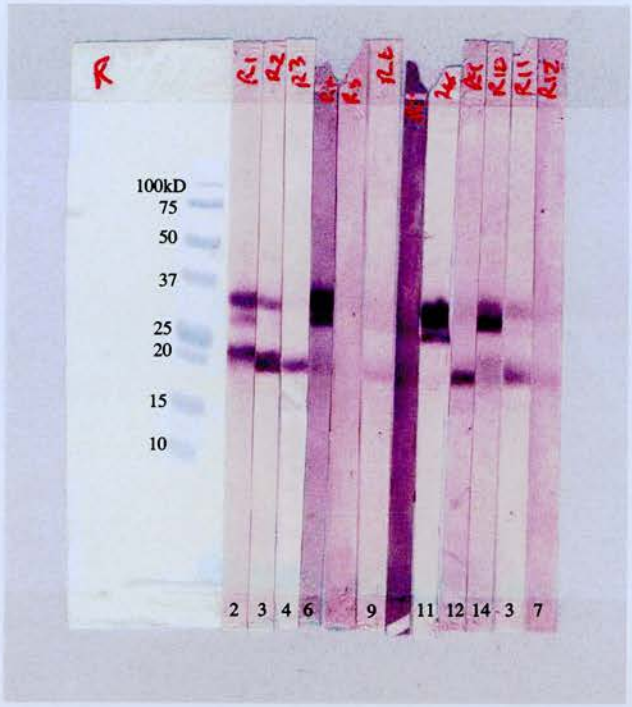

Figure 69 Lectin probing of Pregnyl. For lectin codes see Table 34. [R, reducing conditions; molecular weight markers (M) shown].

Figure 70 Lectin probing of Pregnyl. For lectin codes see Table 34. [R, reducing conditions; molecular weight markers (M) shown].

Figure 71 Lectin probing of JEG3 hCG. For lectin codes see Table 34. [R, reducing conditions; molecular weight markers (M) shown].

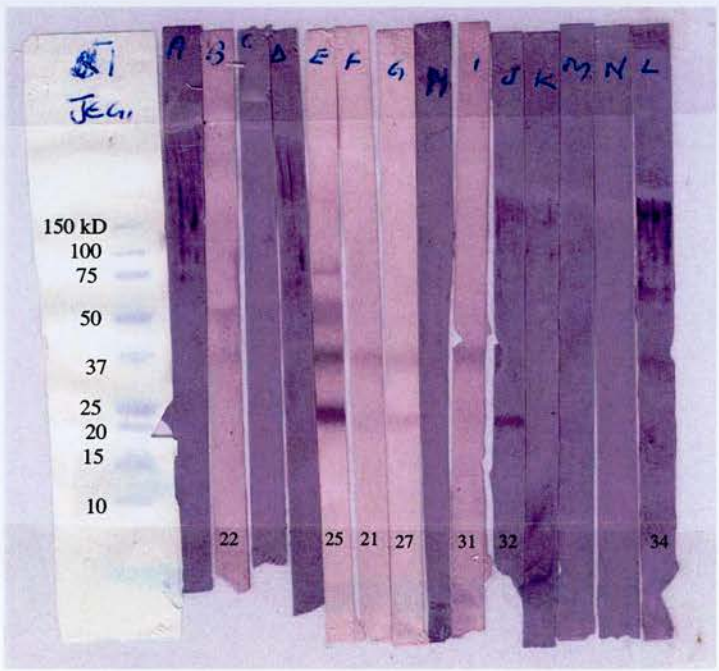

Figure 72 Lectin probing of JEG3 hCG. For lectin codes see Table 34. [R, reducing conditions; molecular weight markers (M) shown].

The lower molecular weights in the Western blots may represent high mannose-type carbohydrate, while the higher molecular weight bands may be due to a more complex-type bi- or triantennary sugar chain branches.

5.2.3 Carbohydrate heterogeneity using different MAbs in hCG from patient samples

Similar studies were performed on human pregnancy samples to determine whether lectin and/or MAb B152 binding studies could helpfully contribute to diagnosis of failing pregnancies. Nine stored urine samples which had been collected at different gestational ages from women in the UAE cohort who had a successful pregnancy outcome were randomly selected. Samples 1-3 were from gestations <6 weeks long, , 4-6 from 7-12 weeks and 7-9 were from the 2nd trimester (Table 35). Three urine samples from molar pregnancies were also selected (Samples 10-12, Table 35). Western blotting was performed using MAbs B152 and 8F11SMA as described previously [Section 2.5.4].

Sample no	Gestation (weeks)	Total hCG (pmol/L)	Dilution	Sample (μL)	Buffer (μl)	Creatinine mmol/L
		Normal pregnancy samples				
1	<6	162548	8.4	100	740	2.4
2	<6	25836	1.3	100	34	2.0
3	<6	19317	1.0	100	0	1.3
4	7- 12	144618	7.5	100	649	1.5
5	7- 12	1279871	66.3	10	653	4.4
6	7- 12	254309	13.2	100	1220	7.7
7	> 13	97340	5.0	100	404	8.2
8	> 13	422079	21.9	10	209	16.0
9	> 13	193703	10.0	100	903	4.4
		Molar pregnancy samples				
10	8	9690	0.5	100	0	1.5
11	9	1449932	75.1	10	740	9.6
12	16	5918619	306.4	4	1220	16.9

Table 35 Urinary hCG and creatinine levels in patient samples that were probed with lectins and MAbs [Section 5.2.4].

In reduced samples, all hCG positive samples containing immunoreactive forms with an approximate molecular weight of 37 kDa were identified as hCGβ or hCGβn, those of 20-25 kDa as hCGβ missing the C-terminal peptide and those of 10-15 kDa as hCGβcf. Except for hCGβcf, all these forms were present in the non-reduced samples as was the intact heterodimer (molecular weight 50 kDa or more).

5.2.3.1 Studies with MAb 8F11SMA

The carbohydrate structures of choriocarcinoma hCG resembled that of normal pregnancy hCG in reduced conditions when probed with MAb 8F11SMA (Figures 73 and 74). Bands with molecular weights of 37 kDa (probably hCGβ) were seen in all normal and molar pregnancy samples (Samples 1-12). Additional bands of 20 kDa and 15 kDa were only seen in the 2nd trimester urine samples and in all molar pregnancy samples and probably represent the C-terminal extension of hCG and hCGβcf, respectively.

5.2.3.2 Studies with MAb B152

Comparison of the gel electrophoretic patterns of hCG from normal pregnancy patient urine and pregnancy samples by immunoblot analysis with B152, prepared in reduced conditions, shows that HhCG is present in urine of pregnant women (Figures 75 and 76). When MAb B152 was used at a 1/5000 dilution to probe the neat urine samples, a 37 kDa band representing HhCGβ, was detected in 8 of the 9 pregnancy samples (Figure 75). Meanwhile, the immunostained band of 37 kD was seen only in 1st trimester urine samples when samples were diluted (Table 35) and a higher concentration of MAb B152 used (i.e. at a dilution of 1/2500) (Figure 76). This supports the results of obtained using the DELFIA assay system, which showed early pregnancy samples contained higher concentrations of HhCG (Sections 3.7.1 and 4.3.2).

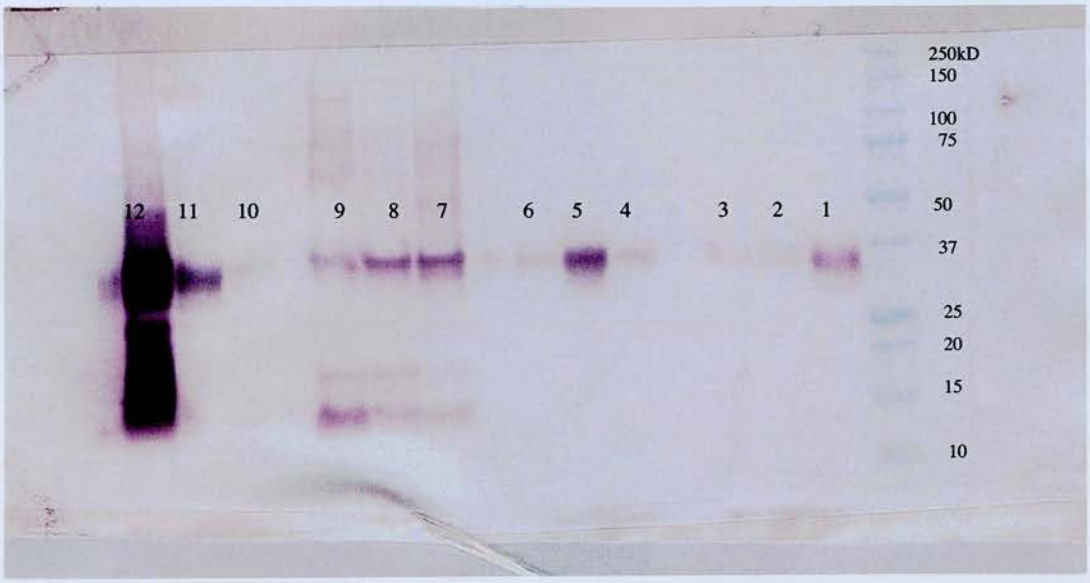

Figure 73 Neat urine Samples 1-12 (Table 35) probed with MAb 8F11SMA (1/5000 dilution). [Reducing conditions; molecular weight markers (M) shown].

Figure 74 Diluted urine Samples 1-12 (Table 35) probed with MAb 8F11SMA (1/2500 dilution). [Reducing conditions; molecular weight markers (M) shown].

Figure 75 Neat urine Samples 1-12 (Table 35) probed with MAb B152 (1/5000 dilution). [Reducing conditions; molecular weight markers (M) shown].

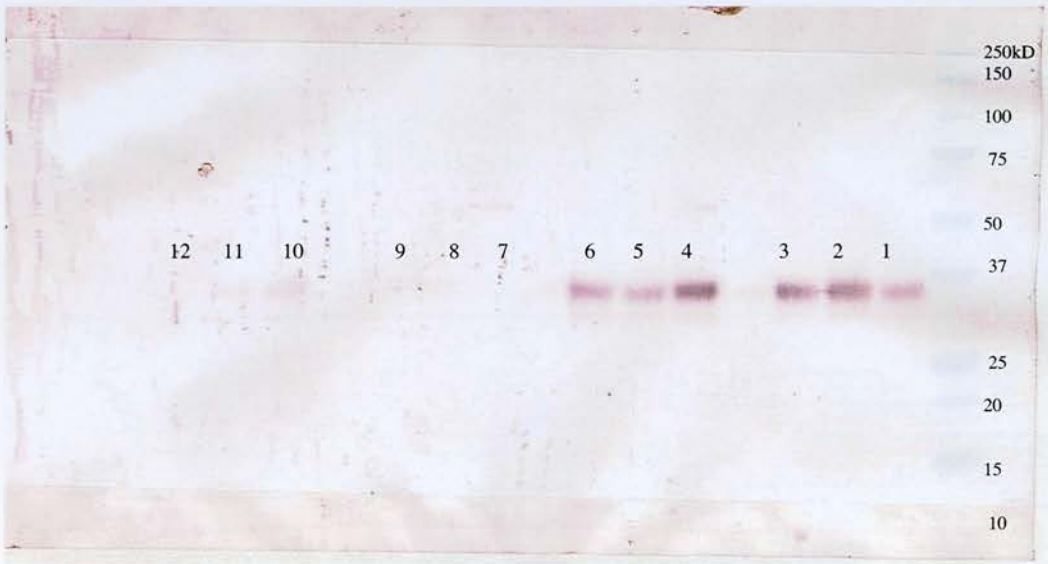

Figure 76 Diluted urine Samples 1-12 (Table 35) probed with MAb B152 (1/2500 dilution). [Reducing conditions; molecular weight markers (M) shown].

5.2.4 Examination of carbohydrate heterogeneity of urine samples from patients with normal or molar pregnancy using lectin-binding

Lectin analysis of urine samples from different gestational stages and using the *Sambucus nigra* agglutinin, *Lotus tetragonolobulus*, Con A, and *Phaseolus vulgaris* leucoagglutinin, in conjunction with an avidin-biotin peroxidase complex, showed heterogeneous binding patterns. Con A and *Sambucus nigra* agglutinin revealed both

high and low molecular weight immunoreactive bands indicating variation in mannose as well as the sialic acid content in the N-linked sugar chains of hCG in most samples irrespective of gestational age (Figure 77 and 78). *Lotus tetragonolobulus* lectin reacted strongly in only two pregnancy samples, suggesting fucose-containing oligosaccharides may not be present in pregnancy urine (Figure 79). Lectin recognizing the tri-antennary oligosaccharides (e.g. *Phaseolus vulgaris* leucoagglutinin) reacted with the majority of normal pregnancy samples, including one molar pregnancy sample, indicating presence of high sugar branches during pregnancy (Figure 80).

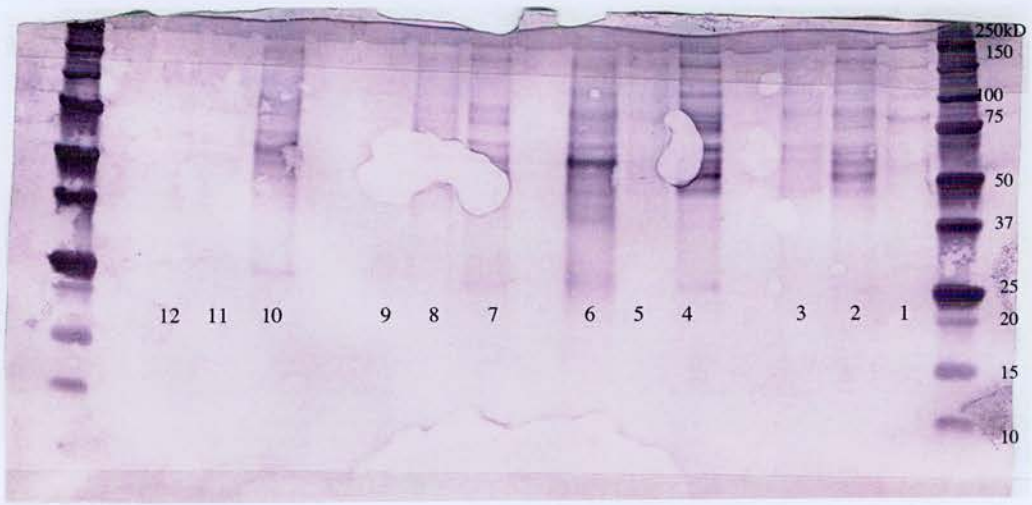

Figure 77 Con A lectin probing of patient urine Samples 1-12 (Table 35). [R, reducing conditions; molecular weight markers (M) shown].

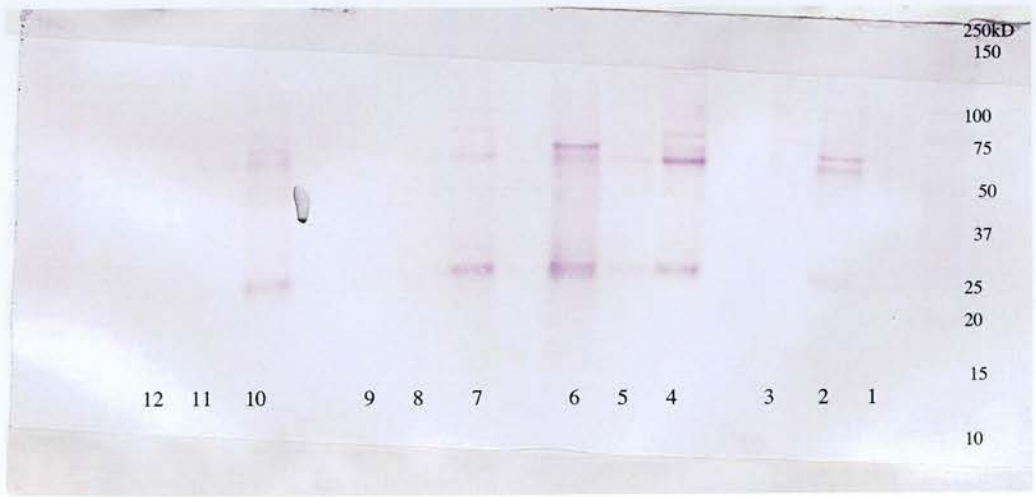

Figure 78 *Sambucus nigra* lectin probing of patient urine Samples 1-12 (Table 35). [R, reducing conditions; molecular weight markers (M) shown].

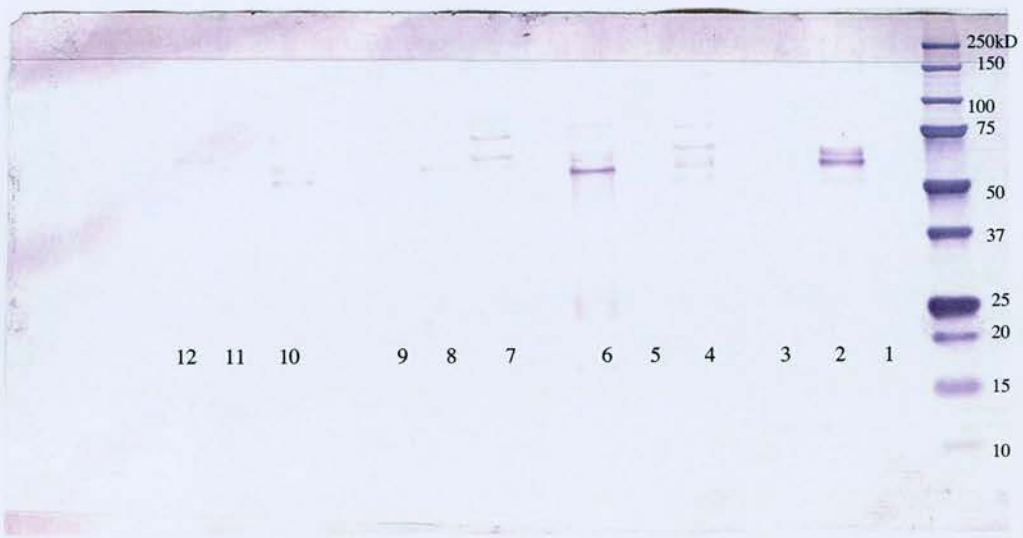

Figure 79 *Lotus tetragonolobulus* lectin probing of patient urine Samples 1-12 (Table 35). [R, reducing conditions; molecular weight markers (M) shown].

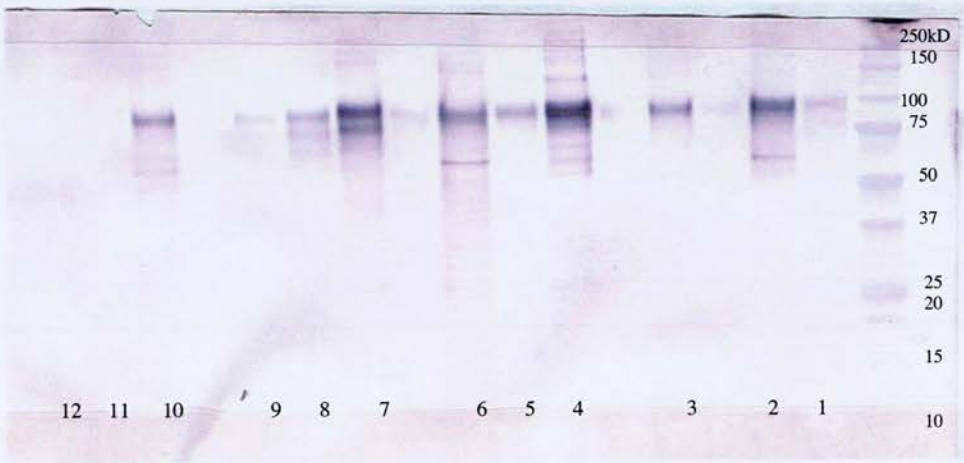

Figure 80 *Phaseolus vulgaris* leucoagglutinin lectin probing of patient urine Samples 1-12 (Table 35). [R, reducing conditions; molecular weight markers (M) shown].

5.3 Discussion of results of molecular heterogeneity studies

5.3.1 Carbohydrate heterogeneity identified by different MAbs in hCG from different sources

The molecular size and carbohydrate content of hCG varies in normal and in pathological conditions. However, Western blotting with MAb B152 (Figures 65 and 66) shows that the same protein bands were detected in hCG from a pregnancy source (Pregnyl) as in JEG3 choriocarcinoma cell lines. This suggests that the protein profile

of hCG does not provide a reliable marker for disease prognosis. Elliot et al. showed that O-linked oligosaccharides of 1st trimester normal pregnancy urine hCG contained 12.3-19% hexasaccharide structures while choriocarcinoma urine hCG contained 48-100% [18]. Site-specific glycan analysis of hCG by liquid chromatography and electrospray mass spectrometry of commercial hCG preparation similar to the one studied here, showed high proportions of core-2 glycan indicating that the preparation was mainly derived from first-trimester urine [77]. The same study showed that N-glycan structures of JEG3-derived hCG resembles that from early pregnancy, including the degree of fucosylation and the content of triantennary glycan structures. The reactivity of both choriocarcinoma-derived hCG and pregnancy-derived hCG with MAb B152 in the current study may be explained in similar manner. As Pregnyl is purified from pooled urines of pregnant women during various gestational ages, these larger sugar chains in Pregnyl may have originated from very early pregnancy urine samples or even from choriocarcinoma hCG contamination within the pooled urine can not be ruled out [84, 208, 527].

In non-reduced samples, B152 showed a band at 50 kDa which may be hCG and a 37 kDa band which may be either the hCG minus the CTP or the nicked/nicked free β-subunit of hCG. It is unlikely that the 37 kDa band represents the hCG minus the CTP since the B152 antibody has shown to specifically detect the hexasaccharide O-linked oligosaccharides on the C-terminal peptide of choriocarcinoma-derived hCG, hence making the presence of hCG CTP mandatory for immunoreactivity [49, 64]. Moreover, a darker band at 37 kDa in JEG3 hCG (Figure 65) confirms the presence of hCGβ which is produced more abundantly in gestational trophoblastic tumours than in normal pregnancy [28, 75, 175, 344, 390, 528].

Interestingly, a higher molecular weight band of 50 kDa was seen in JEG3-derived hCG but not in Pregnyl, in addition to the 30- 37 kDa band seen in both hCG under the reduced condition. This confirms that choriocarcinoma-hCG is hyperglycosylated. As hCG dimer dissociates into free-subunits following the addition of reducing agent, it is unlikely that this 50 kDa band is HhCG. The higher band may represent an oligosaccharide of HhCGβ with variations in its sugar content. As is the case in tumour-derived hCG wherein Core-2 type O-glycans have increased fucosylation of the Asn-13-linked glycan and the presence of these large glycans has been suggested to inhibit the association of the α- and β-subunits thereby increasing the proportion of HhCGβ present [77].

Using MAb 8F11SMA, which recognizes hCGβ, hCGβcf and hCG, the relative intensity of immunostaining of choriocarcinoma and pregnancy hCG differed under the reduced and non-reduced conditions (Figures 63 and 64). While bands at 50, 37, and 25 kDa were seen in both JEG3 and Pregnyl preparations, Pregnyl showed stronger immunoreactivity towards the MAb 8F11SMA. While the nature of this antibody is not yet characterized, it appears that it binds to a conformational epitope present on most molecular forms of hCG. However, the epitope it recognizes may be located in a region near the cystine knot motif of the CTP which is known to be mobile, not visible on the crystal structure of hCG [37], and variably truncated [198]. MAb 8F11SMA may preferentially bind to hCG and hCGβ but not to the nicked forms. This may explain why stronger immunostaining was seen for Pregnyl (Figure 63) than for JEG3 hCG, which is known to be heavily nicked [49, 119, 121, 529].

Reducing conditions are known to destroy the tertiary three-dimensional structure of hCG molecular forms [37, 63, 198]. This may explain the complete absence of immunoreactivity with JEG3 hCG and the reduction in the immunostaining of pregnancy-derived hCG (Figure 64) that is observed when MAb 8F11SMA was used in the current study. Unlike MAb 8F11SMA, MAb B152 binds to HhCG irrespective of the state of nicking of the polypeptide chain and its sialic acid content [63, 67], so immunoreactivity was seen for both choriocarcinoma and pregnancy hCG under both reduced and non-reduced conditions (Figures 65 and 66).

Comparison of gel electrophoretic migration sizes of the hCG from normal pregnancy patient urine belonging to various gestational ages as well as molar pregnancy samples by immunoblot analysis with MAb B152, prepared in reduced conditions, shows that HhCG is present in urines of pregnant women (Figures 75 and 76). When MAb B152 was tested in the neat urine samples with no dilution, i.e. 1/5000 concentration, the 37 kDa band representing HhCGβ was detected in 8 of the 9 pregnancy samples (Figure 75). Meanwhile, the immunostained band of 37 kD was limited to the 1st trimester urine samples when samples were diluted (Table 35) and a higher concentration of MAb B152 (i.e. 1/2500) used (Figure 76). This finding supports the results described earlier for both for the spontaneous pregnancy cohort (Chapter 3) and pregnancies achieved through assisted conception (Chapter 4). This confirms that higher concentrations of HhCG immunoreactivity are observed in the 1st than in the 2nd trimester. This may reflect the limited differential status of trophoblast

cells whereby HhCG is produced by the stem cytotrophoblasts and hCG is produced by differentiated syncytiotrophoblasts [67, 70, 180, 244].

Comparison of the above data (i.e. MAb B152 diluted by 1/5000 in neat urine samples vs. 1/2500 in diluted samples) suggests that using a 1/2500 concentration of MAb B152 in a neat sample may produce stronger immunoreactive bands in early pregnancy samples (<6 weeks gestation) as compared with later pregnancy samples, mirroring the time-shift frame of HhCG production.

In contrast to normal pregnancy, no difference was seen in the immunoreactive bands of molar pregnancy samples using the two different concentrations of MAb B152 as only one of the three samples showed a positive band of HhCGβ at 37 kDa (Figures 75 and 76). This sample belonged to a patient diagnosed with partial mole while the other two were from histologically confirmed complete molar pregnancies.

Interestingly, the intensity of the band detected by MAb B152 did not correlate with the HhCG concentrations of each sample. The positively stained band of the sample from the partial molar pregnancy contained less HhCG (476 pmol/L) than the two complete mole samples (93744 pmol/L and 143042 pmol/L, respectively). This suggests that Western blotting may be detecting additional denatured proteins as it immobilizes the denatured hCG on a membrane surface, and perhaps enhances the binding of MAb to the antigenic region of hCG. Minor molecular differences in hCG glycosylation have little or no influence on the overall immunoreactivity of hCG molecular forms in immunoassay systems [47, 207, 213]. However, problems of inter-assay variations have been reported in assay systems not recognizing HhCG forms that are deficient in their sialic acid content [474]. Heterogeneity of hCG due to differences in the sialic acid content has often been described [31, 82, 498].

Immunoblot analysis with MAb 8F11SMA in reduced conditions, demonstrated bands of 37 kDa molecular weight in all twelve pregnancy samples, presumably representing hCGβ (Figures 73 and 74). Additional molecular weight bands of 20 kDa and 15 kDa were seen in 2nd trimester urine samples and in all molar pregnancy samples, probably reflecting the C-terminal extension of hCG and hCGβcf. This is in accord with literature reports that other hCG molecular forms, including hCGβcf, increase as gestation progresses as well as during trophoblastic disease [111, 113, 530, 531].

Increasing the concentrations of antibody from 1/5000 to 1/2500 revealed additional bands at about 25 kDa position and no bands in the 7-12 weeks in urine pregnancy Samples 4-6. This may be due to differences in nicking of the polypeptide chain

and/or sialic acid content which may be differentially detected by MAb 8F11SMA or to changes in the tertiary conformation.

From the Western blotting results, it can be concluded that the size of Pregnyl hCG resembles that of JEG3 hCG. In reduced samples, variations in the observed staining pattern highlight the heterogeneous nature of hCG and the specificities of the MAbs used but failed to show differences between normal and molar pregnancies, indicating that this approach is not ideal for clinical use.

5.3.2 Carbohydrate heterogeneity identified using lectin binding in hCG from different sources

Lectin-binding patterns have been used to study the invasive potential of cells and neoplastic transformations associated with changes in the expression of various hCG forms. The present study was undertaken to investigate whether qualitative differences in lectin binding patterns can discriminate glycoforms of hCG produced very early during gestation and in molar pregnancies from later pregnancy hCG. The type of sugar and their linkage pattern in oligosaccharides were studied. Binding patterns of 26 lectins to hCG at various gestational stages of normal pregnancy was compared to those for hCG from urine samples from patients with partial or complete hydatidiform mole, and from hCG from choriocarcinoma cell line JEG3 (Table 34).

The lectin-binding pattern of non-reduced hCG in Pregnyl confirms the presence of oligosaccharides containing β-galactosyl N-linked sequences containing either trimers or tetramers of N-acetylgalactosamine and tri-antennary sugar branches (Figures 67 and 68). These bands appeared at molecular weights corresponding to hCGβ and the hCG minus the CTP. Addition of reducing agent not only cleaves hCG into the two subunits but also separates the N-glycosylated amino terminal portion from the O-glycosylated carboxy-terminal portion exposing further binding sites [82, 532]. Hence when Pregnyl was probed with lectins after being reduced, more lectins revealed immuno-positive staining (Figures 69 and 70). The binding pattern in reduced samples confirms the presence of neutral sugars containing core mannose and/or galactose-linked residues, α- and β- galactosyl N-linked sequences containing trimers or tetramers of N-acetylglucosamine and N-acetylgalactosamine, and/or terminal sialic acid molecules. The carbohydrate structures of choriocarcinoma hCG resembled those of normal pregnancy hCG in reduced conditions (Table 1 and Figures 71 and 72). While the sugar chains of both purified hCG preparations studied here are

sialylated, Pregnyl hCG showed stronger binding to sialic acid binding lectins (Table 34) as compared to JEG3 hCG. This is in agreement with other structural studies of hCG during pregnancy, Down syndrome, trophoblastic diseases as well as other malignant tumours, showing HhCG to be mostly deficient in sialic acid [18, 20-22, 24, 27, 28, 55, 78, 131, 474, 533, 534]. It is suggested that the deficiency of terminal sialic acid residues allows N-acetylglucosaminyl transferases to initiate the addition of bi- or trisaccharide sugars to the oligosaccharide side chains, making the molecule hyperglycosylated [535].

Con A commonly binds to asparagine-linked oligosaccharides in both α- and β-subunits ending with mannose residues, but these are substituted with N-acetylglucosamine, galactose, and sialic acid antennae the binding is weakened [27]. Even if pregnancy hCG contains mannose structures at both glycosylation sites, failure to bind with Con A lectin may indicate presence of other complexed sugars (Figures 67-70). The opposite mechanism maybe operating in the JEG3 hCG wherein higher sugar branches contribute to tight interaction of hCG with Con A (Figure 71).

Lectin recognizing core fucosylated, bi- or tri-antennary oligosaccharides (*Lotus tetragonolobulus* +ve) was only seen in JEG3 hCG. This supports previous reports that the activity of fucosyl transferase is increased in invasive mole and in choriocarcinoma [18, 121, 402]. Similarly, the presence of triantennary sugar chains in JEG3 hCG (*Phaseolus vulgaris* leucoagglutinin +ve) indicates enhanced activity of N-acetylglucosaminyl transferase IV, which is responsible for the formation of the GlcNAcβ-4Manα1-3 sugar chain group [402]. The presence of fucosylation has shown to alter carbohydrate mobility in the SDS-PAGE system by preventing binding of the lectins with affinity for α-/β-linked N-acetylglucosamine [536]. This may explain why some of the lectins in the current study failed to react with α-/β-linked N-acetylglucosamine sugars in JEG3 hCG although they did with Pregnyl hCG. Jacalin lectin which is specific for O-linked sugar chains on the CTP bound to both Pregnyl (Figures 67 and 69) and JEG3-hCG (Figure 71) suggesting that this sugar is present in both pregnancy and choriocarcinoma hCG.

Probing of MAb B152 in patient samples (Table 35) (Figures 75 and 76), and in Pregnyl (Figure 65) and JEG3 hCG (Figure 66), resulted primarily in a band of 37 kDa molecular weight, confirming that the B152 epitope lies within the CTP region of the β-subunit [49, 63, 64].

Although hCG and hCGβ are used to assess the progression of trophoblastic disease, they do not always correct disease progression or regression [217, 390, 537-542]. For this

reason, lectin-binding pattern was evaluated as a possible alternative method for early detection of disease. Lectin analysis of urine samples from different gestational stages and from normal and molar pregnancies showed different hCG binding patterns (Figures 77-80). *Sambucus nigra* agglutinin reacted with pregnancy samples irrespective of the gestational age, indicating variation in the sialic acid content (Figure 78). The sialylation pattern observed for hCG reflects the length of gestation [78, 82, 474], pathological conditions (e.g. gestational trophoblastic disease or Down syndrome) [29, 78, 475, 543], source (e.g. urine, serum, tissue or cell culture), [160, 498, 532] as well as the methods used for detection [29, 77, 78, 82, 534].

Con A showed high as well as low molecular weight immunoreactive bands indicating variation in the mannose content in the N-linked chain of the hCG in pregnancy samples irrespective of their gestational age (Figure 77). During the glycosylation process, high mannose sugar residues are first attached to glycosylation sites on the peptide chain which are then modified to form complex type structures that give rise to final "matured" glycosylated protein [33, 90]

Compared to normal pregnancy, in hCG produced during trophoblastic diseases levels of N-linked fucosylated biantennary oligosaccharide, fucosylated triantennary oligosaccharide, and tetrasaccharide core structure are increased due to higher activity of branching enzymes [18, 24, 35, 61, 402]. Here, the triantennary branching on the β-subunit was only evident for one partial molar pregnancy sample as it reacted weakly with *Phaseolus vulgaris* leucoagglutinin (Figure 80).

Hydatidiform mole is generally a benign lesion, but some moles show more malignant characteristics such as invasion in the surrounding tissues and metastasis leading to alterations in the levels and structure of hCG sugar chains associated with the pathological process [28, 34, 75, 175, 217, 243, 336, 349, 352, 537, 538, 542, 544-547]. The importance of identifying patients at risk of developing gestational trophoblastic disease following the evacuation of hydatidiform mole has been highlighted in earlier chapters. The lack of qualitative differences between the normal pregnancy and molar pregnancy samples found in the current study using either MAb B152 or lectin binding profiles indicates that this method is not suitable for identifying women at risk of developing persistent disease. Kelly et al. developed a lectin-based immunoassay comparing the glycosylation patterns of hCG in urine specimens from normal pregnancy, invasive mole, choriocarcinoma, and male germ cell tumours using a panel of eight lectins. Two lectins (*Galanthus nivalis* agglutinin and *Maackia amurensis* lectin)

preferentially bound to pregnancy-derived hCG, while WGA preferentially bound to hCG from male germ cell tumours and malignant gestational trophoblastic neoplasia [78]. Higher intensity of binding of Jack fruit lectin was noted in patients with persisting gestational trophoblastic disease and showed close correlation with the regressing pattern of serum hCG [547]. Lectin analysis of the receptors in normal placenta and trophoblastic disease showed increased binding of Con A and WGA in samples from patients with hydatidiform mole, invasive mole and choriocarcinoma as compared to normal pregnancy placenta. It was concluded that the reactivity with ConA and WGA was generally associated with increased growth and proliferation of trophoblasts rather than malignant transformation of cells [532]. Normal placenta, partial and complete mole showed pronounced binding with peanut agglutinin after neuraminidase treatment, as compared to invasive mole and choriocarcinoma. It was suggested that in some malignant trophoblasts, sialic acid is missing from the terminal carbohydrate groups resulting in the exposure of N-acetylgalactosamine. Any hyperproliferation of placental cells during various stages of pregnancy may be associated with a loss of sialic acid content thereby exposing the glycoprotein side chains [532].

Increased triantennary structures linked to Asn-30 as well as fucosylation of the Asn-13-bound glycan were found in the β-subunit of cancer-derived hCG when compared to hCG from normal pregnancy [77]. This observation is confirmed in the current study where using lectins with affinity for fucose-linked sugar chains, positively stained bands were seen in JEG3 hCG (Figure 71) but not Pregnyl (Table 34). Probing of pregnancy urine with *Lotus tetragonolobulus* lectin showed darker stained bands only in 1st trimester samples (Figure 79) This contradicts previous reports suggesting that the proportion of highly branched, fucosylated oligosaccharides increases as gestation progresses [27, 31].

In summary, Western blotting experiments were carried out to identify hCG bands by means of antibody-antigen interaction. The results presented here are generally in good accord with evidence in the literature. The present results provide information about oligosaccharide composition of pregnancy hCG and confirm the complex structure and heterogeneity of the carbohydrate structure of hCG [18, 21, 24, 35, 61, 66, 67, 77, 402, 532]. The lack of apparent qualitative differences using MAb B152 or lectin binding profiles in patient samples may reflect the low concentrations of relevant sugar residues and indicates the need for more sensitive detection methods.

CHAPTER 6. FUTURE DIRECTIONS AND SUMMARY

6.1 Future Directions

6.1.1 Pregnancy test and early pregnancy outcome

This study has confirmed that the majority of total hCG immunoreactivity in serum and urine samples during early pregnancy is due to HhCG. Most commercially available hCG tests [69, 340, 548, 549] have been calibrated with hCG and not HhCG and do not optimally recognize the HhCG. Since 2006 Quest Diagnostics Inc. have owned the patent rights to MAb B152 [550], but the official commercial release of a HhCG assay system is still awaited.

The availability of an assay that appropriately detects HhCG is not only important in the detection of pregnancy, but can be extended in monitoring of early pregnancy outcome. Both, single cut-off value as well as doubling time, should be further explored not only to distinguish normal pregnancies from failing pregnancies, but also can be used as a better discriminator between ectopic pregnancy and spontaneous miscarriage. Efforts should be made to define the 'discriminatory zone' for HhCG, like the one available for hCG [312, 505, 551], in order to make a quick accurate early diagnosis of ectopic pregnancy even when the sac is not visualized on ultrasound.

Placenta accreta, placenta increta and placenta percreta represent a spectrum of abnormal implantation by the placenta, in which there is abnormal attachment of the placenta to the uterine wall that may lead to massive obstetrics hemorrhage and the need of extensive life-saving surgical interventions such as hysterectomy and ligation of major pelvic vessels [552]. HhCG may be used for early recognition of the condition which may improve the outcome, since it provides the obstetrician the opportunity to deal more effectively with this obstetrical emergency.

It is not easy to assign cause and effect from the data presented in this thesis, it is tempting to speculate that decreased levels of HhCG in the placenta may lead to early pregnancy failures and spontaneous miscarriages. Future research will need to address the exact role of HhCG in implantation and its mechanism of action throughout pregnancy in order to develop new therapeutic strategies to prevent and treat pregnancy-related disorders such as spontaneous miscarriages and pre-eclampsia. Application of MAbs against HhCG for passive immunization, in cases of emergency

contraception, could also be explored. The present lack of availability of highly purified and well-characterized HhCG preparations limits further progress in this area.

6.1.2 HhCG as a tumour marker and treatment of malignancy

A role for HhCG in promoting both growth and invasion by pregnancy cytotrophoblast and choriocarcinoma cells in vivo and in vitro has been shown [74, 253]. HhCG may be a specific promoter or stimulant of cytotrophoblast and choriocarcinoma invasion *in vitro* and choriocarcinoma growth and development *in vivo* and blockage of HhCG with MAbs may obstruct choriocarcinoma tumourigenesis and growth and development *in vivo*. MAbs with the same specificity, or appropriately modified mouse antibodies, or antagonists of HhCG could be clinically useful as a treatment for choriocarcinoma and gestational trophoblastic disease. They could also be clinically useful in the prevention of persistence of hydatidiform mole or of recurring trophoblastic diseases. HhCG might have potential as a vaccine to promote endogenous production of human antibodies. Potential angiogenic function should also be explored for HhCG, since HhCG-antagonists could serve as a potential approach for angiostatic therapy in malignancy.

6.2 Conclusions

Even with the results of the present study, together with all the currently available data in the literature, it is not possible to predict whether maternal screening for obstetrics complication with HhCG will become a clinical reality. The test undoubtedly has potential for detecting early pregnancy failures and spontaneous miscarriages. Its true clinical value as a screening marker remains to be determined. However it is tempting to speculate that measurement of HhCG could provide a 'contingency screen' to follow the outcome of early pregnancy, and, in combination with other tests, an 'integrated screen' for following up the prognosis of later gestation, both of which need to be verified in future prospective studies.

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Appendices

MINUTES OF ETHICAL & INTERPERSONAL COMMITTEE MEETING

DATE: 1/6/2003 TIME: 1300 Hrs. VENUE: CONFERENCE ROOM-NEW ADMINISTRATION CHAIRPERSON: DR. GERALD STILES

PRESENT: Prof. H. Grundsell, Mrs. Linda Jackson

APOLOGY: Prof. Mohamed Farrakha, Dr. Amin El Gohary

ABSENT: Dr. Mohamed Saeed, Dr. Claes Henning, Dr. Idris El Hag

MINUTES TAKEN: Dr. Gerald Stiles

MINUTES OF PREVIOUS MEETING: Not discussed at this session

NEXT MEETING: Not determined

Agenda No	Title/Topic	Discussion/Agreement/Action	Person Responsible for	Target Date
1.	To review a prospective study on the evaluation of Hyperglycosylated Human Chronic Gonadotrophin (H-hCG) as a Predictor of Adverse Pregnancy Outcome	Principal Investigator: Dr. Nagla Kazim Co-Applicant: Prof. Hans Grundsell Project undertaken in conjunction with the Faculty of Medicine of the University of Edinburgh. See letter attached. Details of the study are outlined in the attached document. Two minor alterations were made to the text on review by the members of the committee. Assurance was given that there was an Arabic version of the consent form.		

Contd./2

Agenda No	Title/Topic	Discussion/Agreement/Action	Person Responsible for	Target Date
		Conclusion: Having reviewed all aspects of this project, the committee concluded as there were neither ethical nor scientific reasons why the study should not proceed.		

DR. GERALD STILES
Chairman
Ethical & Interpersonal Committee
Mafraq Hospital

PROF. HANS GRUNDSELL
Senior Consultant & Head
Obs & Gynae Department
Mafraq Hospital

MRS. LINDA JACKSON
Director of Nursing
Nursing Department
Mafraq Hospital

Consent Form For Defining the Screening and Diagnostic strategies of Hyperglycosylated hCG in detection Of Pregnancy Related Disorders

I agree to participate in the project for detecting the screening and diagnostic efficiency of hyperglycosylated hCG in detecting pregnancy related disorders: a study to help identifying high risk pregnancies before the onset signs and symptoms of any disorder. I understand that I will complete a brief questionnaire about my health and obstetrical history. I agree to provide my urine and blood samples for the above mentioned project. I understand that the first urine and blood samples will be taken during an early ante natal booking (i.e. between 8-14 weeks) followed by the second urine and blood samples, which would be taken at 15-20 weeks of my pregnancy. I am aware that these samples will be then stored and analyzed once the pregnancy outcome is available. My only discomfort will be related to the needle prick for drawing the 3 cc blood, and the most common adverse is bruising around the vein from where the blood sample is drawn.

I understand that my participation in the study is entirely voluntary. All information gathered for this study will be used by the investigators exclusively for the research purposes and will not be used to generate a profit. I will not be identified in any published report. I am free to refuse to participate or withdraw from the study at any time, without jeopardizing my future ante natal care. If I have any question, I may contact **Dr. Nahla Kazim, Mafraq Hospital,** on telephone number 02-5031537 and Pager no. 208.

I agree to participate in the project defining the efficiency of H-hCG in detecting pregnancy related disorders.

.....	.....	.....
Subject Name	**Subject Signature**	**Date**
.....	.....	.....
Witness Name	**Witness Signature**	**Date**

Dr. Nahla Kazim
G.P. / Obstetrics and Gynecology Dept.
Mafraq Hospital

Characteristics of study group

Patient ID [ ] File no. [ ]

Name [ ]

DOB [ ] Age [ ]

Nationality [ ] Contact no. [ ]

Height [ ] Weight [ ] BMI [ ]

Blood Group [ ] Hb [ ] BP [ ]

LMP [ ] EDD [ ]

G [ ] P [ ] A [ ]

Personal habits

[ ]

Family history

[ ]

[ ]

Medical history

Surgical history

Obstetrics history

U/S findings

Remarks

Pregnancy Complications

Early Pregnancy

- ☐ Hyperemesis Gravidarum
- ☐ Threatened abortion
- ☐ Missed abortion
- ☐ Incomplete Abortion
- ☐ Spontaneous abortion
- ☐ Ectopic pregnancy
- ☐ Molar Pregnancy

Others

Comments

Late pregnancy

Gestational diabetes

☐ On diet ☐ On insulin

Hypertensive disorders

☐ PIH ☐ Pre-eclampsia ☐ Eclampsia

Preterm

☐ Spontaneous ☐ Induced

☐ IUGR

☐ LBW

☐ Macrosomia

☐ Abruption

☐ Still birth

☐ Neonatal death

Others

Comments

Pregnancy Outcome

Gestational age at delivery ☐

☐Spontaneous
☐Induced

Mode:
☐Vaginal ☐CS ☐Forceps ☐Vacuum

☐Alive
☐Dead

☐Male
☐Female

Birth Weight ☐
Apgar Score ☐/10, ☐/10

Placenta

Remarks

SAMPLE DATA

A) 1st sample

Gestational age

Code

Date Collected

Next Visit

B) 2nd sample

Gestational age

Code

Date Collected

Remarks

IVF Study Protocol

Lothian NHS Board

Deaconess House
148 Pleasance
Edinburgh
EH8 9RS
Telephone 0131 536 9000
Fax 0131 536 9009
www.nhslothian.scot.nhs.uk

NHS
Lothian

Deaconess House
148 Pleasance
Edinburgh
EH8 9RS

Telephone: 01315369050
Facsimile: 0131 536 9346

31 January 2006

Prof. Andrew A Calder
Head of Department- Obstetrics and Gynaecology
Department of Reproductive and Developmental Sciences
Simpson centre for Reproductive Health
51 Little France Crescent
EH16 4SA

Dear Prof. Calder

Full title of study: **Hyperglycosylated hCG(H-hCG) Levels following Embryo Transfer(ET): A potential marker of successful implantation and uneventful pregnancies?**

REC reference number: **05/S1101/33**

Thank you for your letter of 11 October 2005 received 25 January 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered by the chair on behalf of the LREC1.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	1 updated	01 July 2005
Investigator CV		
Protocol	1	01 August 2005
Letter from Professor		07 October 2004

Peer Review		07 December 2004
GP/Consultant Information Sheets		11 October 2005
Participant Information Sheet	4.2	11 October 2005
Participant Consent Form	1	01 August 2005
Response to Request for Further Information		11 October 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/S1101/33 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Chair

Email: stephanie.lamb@lhb.scot.nhs.uk

Enclosures:

Standard approval conditions [SL-AC1 for CTIMPs, SL-AC2 for other studies]
Site approval form

SSI - list of approved sites

Lothian Local Resear[ch] Ethics Committee 01

LIST OF SITES WITH A FA[VO]URABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the mai[n] tEC to the Chief Investigator and sponsor with the favourable opin[ion] following subsequent notifications from site assessors. For issue 2 onwards, all [sit]es with a favourable opinion are listed, adding the new sites appro[ved]

REC reference number:	05/S1101/33	**Issue number:**	1	**Date of issue:**	31 Janu[ary]

Chief Investigator: Prof. Andrew A Calder

Full title of study: Hyperglycosylated hCG(H-hCG) Levels fo[l]lowing En[dr]yo Transfer(ET): A potential marker of successful implantation an[d] pregnancies?

This study was given a favourable ethical opinion by the chair on behalf of L[othian] Local Research Ethics Committee 01 on 31 January 2006. The fa[vourable opinion] is extended to each of the sites listed below. The research may commence at eac[h] NHS site when management approval from the relevant NHS co[mpany] has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes (1)*
DR NAHLA A R KAZIM	PhD Student	The University of Edinburgh	Lothian Local Research Ethics Committee 01	31/01/2006	

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/Administrator)
(delete as applicable)

..... (Name)

(1) *The notes column may be used by the main REC to record the early clo[sure] or withdrawal of a site (where notified by the Chief Investigator suspension of termination of the favourable opinion for an individual site, [or] any other relevant development. The date should be recorded.*

University Hospitals Division

NHS
Lothian

ROYAL INFIRMARY OF EDINBURGH
51 Little France Crescent, Edinburgh, EH16 4SA

RESEARCH & DEVELOPMENT OFFICE

Tel: 0131 242 3330
Fax 0131 242 3343
Email
R&DOffice@luht.scot.nhs.uk

Director:
Dr Heather A Cubie
FRCPath

R&D Project Manager:
Rachel Smith
PhD

Commercial Research Manager:
Douglas Young
PhD MBA

Accountant:
Ms Sheevaun McIntyre
BAcc

Assistant Accountant
Mr Neil McLean

PA/Office Manager:
Mrs Glynis Omond

Administrative Assistant:
Miss Emma Lewis

HAC/GO/approval/2e,3,3b+mta

2 November 2005

Dr Nahla Kazim
PhD Student
Obstetrics/Gynaecology
Simpson Centre for Reproductive Health
RIE

Dear Dr Kazim

MREC No:	**N/A**
CRF No:	**N/A**
LREC No:	**05/S1101/33**
R&D ID No:	**2005/R/RM/16**
Title of Research	*Hyperglycosylated hCG levels following embryo transfer*
Protocol No/Acronym:	**N/A**

The above project has undergone an assessment of risk to the Division and review of resource and financial implications. I am satisfied that all the necessary arrangements have been set in place and that all Departments contributing to the project have been informed.

I note that this is a single centre study sponsored by **University of Edinburgh.**

Use of Tissue or Samples

- The study involves the use of patient tissue or samples. You must be familiar with NHS Lothian's Tissue Policy and abide by its conditions and also with all regulations in place at the time. Approval is subject to the prevailing legal requirements.
- Approval for the use of tissue is restricted to the protocol associated with this application, but may include additional collaborators within University of Edinburgh. Collaborators who are not named in the original protocol require to be notified to local REC.
- If material is to be transferred to academic collaborators outwith University of Edinburgh or to any commercial entity then a material transfer agreement must be obtained from the R&D Office and signed by all relevant parties prior to transfer of the material. Such collaborations must be fully discussed with the R&D Office.
- I note that additional samples will be taken for the study and that this will be done with the patient's explicit consent.

On behalf of the Chief executive and Medical Director, I am happy to grant management approval from NHS Lothian - University Hospitals Division to allow the project to commence, subject to the approval of the appropriate Research Ethics Committee(s) having also been

obtained. You should note that any substantial amendments must be notified to the relevant Research Ethics Committee and to R&D Management with approval being granted from both before the amendments are made.

Please note that under Section A, Q35, NHS Lothian provides indemnity for negligence for NHS and Honorary clinical staff for research associated with their clinical duties. It is not empowered to provide non-negligent indemnity cover for patients. Lothian University Hospitals Division does not provide indemnity against negligence for healthy volunteer studies. This is the personal responsibility of both NHS and honorary employees and is usually arranged with a medical defence organisation or through the University of Edinburgh.

This letter of approval is your assurance that the Division is satisfied with your study. As Chief Investigator or local Principal Investigator, you should be fully committed to your responsibilities within the Research Governance Framework for Health and Community Care, an extract of which is attached to this letter.

Yours sincerely

Dr Heather A Cubie
R&D Director

enc	**Research Governance Certificate**	**✓ (to be signed and returned)**
	NRR authorisation	**✓ (to be signed and returned)**
	Tissue Policy (if applicable)	**✓**
	MTA (if applicable)	**✓ (to be signed and returned)**

cc ***Administrators, Research Ethics Committee***
Professor Andrew Calder, Obstetrics/Gynaecology, RIE
Joyce Rogers, Combined Labs, RIE

Patient ID ☐ Initials ☐

DOB ☐

Age ☐

Day of Embryo Transfer
- ☐ D 2
- ☐ D 3

Indication for treatment
- ☐ Tubal factor
- ☐ Endometriosis
- ☐ Male factor
- ☐ Unexplained
- ☐ Others

Type of treatment
- ☐ IVF
- ☐ ICSI

No. of Oocytes Retrieved ☐

No. Fertilized ☐

Embryo type
- ☐ Fresh
- ☐ Frozen

Embryo quality
- ☐ Stage ☐ Grade
- ☐ Stage ☐ Grade

Pregnancy outcome
- ☐ None (Negative pregnancy test)
- ☐ Biochemical pregnancy
- ☐ Ectopic pregnancy
- ☐ Positive fetal heart on U/S x ☐ (* No. of fetal hearts)
- ☐ Miscarriage at ☐ weeks

Remarks
☐

Delivery outcome
Delivered at ☐ Weeks

☐M/☐F ☐ Grams

☐M/☐F ☐ Grams (* Applicable for multiple births)

Others (i.e. chromosomal anomaly or any other complications)

Procedure Details

Date of oocyte recovery

Date of Embryo transfer

Date of hCG test

hCG result

11/10/2005
Version 4.2

PATIENT INFORMATION SHEET

Determination of Hyperglycosylated Human Chorionic Gonadotrophin (H-hCG) levels in Implantation and Early Pregnancy following Embryo Transfer

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please do not hesitate to contact on the number given below if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?
H-hCG is a hormone that is abundant in very early pregnancy (i.e. 3-4weeks). It is produced by placental cells which are necessary for successful implantation. The role of H-hCG in implantation is unclear, but its levels may help doctors to identify successful implantation.
We thus aim to study levels of H-hCG during early pregnancy, as a possible marker for successful implantation and uneventful pregnancy.

Why have I been chosen?
As you are attending the assisted conception unit for the procedure of Embryo Transfer, your follow up blood samples will provide useful information about levels of H-hCG during implantation period.

What will happen to me if I take part?
The IVF clinic will ask you to come back 2 weeks after the procedure for a Pregnancy Test. If you agree to participate, then an extra two teaspoonfuls of blood sample will be drawn for this study.

University Hospitals Division

NHS Lothian

Centre Number:
Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Hyperglycosylated hCG Levels Following Assissted Conception

Name of Researcher: Nahla Kazim

Please initial box

1. I confirm that I have read and understand the information sheet dated 01/08/2005 (version 4.1) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [Assisted Conception Unit] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

SDS0210M P.I.S. (February 2001)

Will my taking part in this study be kept confidential?
All information that is collected about you during the course of the research will be kept strictly confidential. Your blood samples collected will be appropriately coded and will have no details identifying you.

Who has reviewed the study?
The local Research Ethics Committee has reviewed the study.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. Should you decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

Will my GP be informed?
If you agree to participate, then a letter will be sent to your G.P informing them about your participation in this study.

Contact for Further Information
If you have any queries, do feel free to contact Dr J Thong (0131-2422446), Dr Nahla Kazim (0131-2422693) or any other member of the staff in the unit looking after you.

Thank you for your time

OBSTETRICS and GYNAECOLOGY
Reproductive and Developmental Sciences
Simpson Centre for Reproductive Health

Royal Infirmary
51 Little France Crescent
Edinburgh EH16 4SA

Direct Dial 0131 242 2694
Switchboard 0131 650 1000
Fax 0131 242 2686
Email a.a.calder@ed.ac.uk
Email K.Wilkinson@ed.ac.uk

11/10/05
05/s1101/33

Dr .....

Address .....

Dear Dr .....,

Re: Your patient .....

Study title: Hyperglycosylated hCG levels following Assisted Conception

Your patient has recently agreed to participate in the above study which is taking place in the Assisted Conception Unit of Edinburgh Fertility and Reproductive Endocrine Centre. Details of the study are outlined in the enclosed patient information sheet.

If you require any further information please do not hesitate to contact me on 0131-2422693 or any member of assisted other member of the staff in the assisted conception unit.

Yours sincerely,

Dr Nahla Kazim (M.B.B.S, MSc)

HEAD OF DIVISION Professor A A Calder